PREDATION AND PARASITISM OF THE KAMEHAMEHA BUTTERFLY (*VANESSA TAMEAMEA*) ON OAHU ISLAND

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ABSTRACT

Hawaii's official state insect, the Kamehameha butterfly (*Vanessa tameamea*), is one of only two butterflies native to the Hawaiian Islands. Recently, this iconic butterfly has experienced a population decline and is not present in areas where it once thrived. Since little research has been previously conducted on the butterfly, past studies on other declining Lepidoptera species were examined to gain insight into what factors may be affecting Kamehameha butterfly populations. A rearing methodology for *V. tameamea* was also developed because sentinel eggs and larvae were needed for field trials, and to provide a basis for future conservation programs. Predation and parasitism rates of *V. tameamea* were estimated using sentinel eggs and larvae that were deployed for three days under various treatments at four sites on Oahu, Hawaii. Data was analyzed using risk assessment analysis and binomial logistic regression. Bird predation and ant predation varied by site, and parasitism was detected at only one site. In conclusion, results showed that the butterfly could potentially be reintroduced to areas where they are currently extirpated, if certain controls are implemented, and if other factors (such as host plant scarcity or habitat quality) are not limiting.

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CHAPTER 1

A REVIEW OF PRIMARY CAUSES OF BUTTERFLY DECLINE ON ISLANDS AND IN CONTINENTAL ECOSYSTEMS

Introduction

Butterflies, are more charismatic and well known than most moths and other insect groups. Many species of butterfly are in decline or considered at risk for extinction The International Union for the Conservation of Nature (IUCN) and the United States Fish and Wildlife Service (USFWS) currently list over 200 butterfly species as being threatened on some level (IUCN, 2017; Table 1.2). In the Hawaiian archipelago there are roughly 1000 native species of Lepidoptera (Nishida, 2002), but only two of these are butterflies: the Kamehameha butterfly, *Vanessa tameamea* Esch. (Lepidoptera: Nymphalidae) and Blackburn's blue, *Udara blackburni* Tuely (Lycaenidae) (Zimmerman, 1958). Recent observations suggest that the Kamehameha butterfly is in decline (Haines, 2014). The major factors cited as threats to butterfly populations worldwide include: invasive plants (Braby, 2010), introduced predators and parasitoids (Gripenberg et al., 2011), grazing by ungulates (Saarinen et al., 2005), habitat alteration by humans (Fox, 2013), scarcity of larval host plants, poor nectar sources (Schultz & Dlugosch, 1999), uncontrolled or controlled fires (Armstrong et al., 2013; Swengel et al., 2011), and global climate change (Braby, 2010). The reasons for the decline of the Kamehameha butterfly are unclear. In Hawaii, the high number of introduced ant species (and lack of any native ants) as well as numerous introduced birds and generalist parasitoids, may be a significant cause of decline for this endemic species.

Herein, I review the factors contributing to the decline of butterfly species worldwide and compare patterns of endangerment between continental and island ecosystems in order to better understand the potential factors contributing to the decline of the Kamehameha butterfly.

Compiling and Comparing Threats on Islands and Continents

In order to identify the primary factors causing butterfly declines, I reviewed the information available on all butterfly species listed by the IUCN and USFWS. When investigating butterfly threats, only data from within a species' native range were considered. Species were divided into island species and continental species, with species native to both continental and island land masses classified as continental.

The Environmental Conservation Online System (ECOS) of the USFWS provided a list of all federally endangered and threatened butterfly species in the United States, along with their distribution and associated Federal Register documentation. The Xerces Society website (http://www.xerces.org/) also summarized information regarding important threats associated with specific butterfly species listed by the USFWS, and sometimes included more current information than what was listed in the Federal Register.

Information was also compiled from the IUCN Red List website (IUCN, 2017). Because the IUCN Red List classifies species under 10 different status assessments ranging from "Extinct" to "Least Concern" and includes over 600 butterfly species worldwide, search options were used to narrow down the number of species. The search was limited to butterfly species with the assessment status "vulnerable", "endangered", "critically endangered", or "extinct". After applying the search options mentioned, the butterfly families Hesperiidae, Lycaenidae, Nymphalidae, Papilionidae, and Pieridae were represented. Threats were compiled from each butterfly species' individual webpage, and if threat information was not available, no threat was recorded. The 28 butterfly species listed by USFWS were combined with those listed by IUCN. The IUCN and USFWS categories of "endangered" were combined together for the purposes of this review. Species listed as "threatened" by USFWS were categorized with species listed as "vulnerable" by IUCN.

Table 1.1. Federally Threatened (T) and Endangered (En.) butterflies currently listed by USFWS and the threats reported for each species. Habitat alteration is considered to be any direct impact on butterfly habitat by humans (e.g. Urban expansion, forestry, agriculture, mining, logging). Species whose distributions included both continents and islands are considered to be continental. Reference codes: 1. Xerces Society; 2. Federal Register document for respective species from USFWS Environmental Conservation Online System.

Family	Species	Common names (Eng)	Island or Continent	Status	Disease	Parasites and Predators	Invasive Plants	Habitat alteration	Climate change	Grazing of Host	Fires	Suppresion of Natural Fires	Collectors	Pesticides	Other	Reference
LYCAENIDAE	*Callophrys mossii bayensis*	San Bruno Elfin butterfly	Co.	En.			X	X								1
LYCAENIDAE	*Cyclargus ammon*	Nickerbean blue Butterfly	Co.	T			X	X	X	X			X			2
LYCAENIDAE	*Cyclargus thomasi bethunebakeri*	Miami Blue butterfly	Co.	En.				X					X	X		1
LYCAENIDAE	*Euphilotes battoides allyni*	El Segundo Blue butterfly	Co.	En.			X	X								1
LYCAENIDAE	*Euphilotes enoptes smithi*	Smith's Blue butterfly	Co.	En.			X	X		X						1
LYCAENIDAE	*Glaucopsyche lygdamus palosverdesensis*	Palos Verdes Blue butterfly	Co.	En.			X	X				X				1
LYCAENIDAE	*Hemiargus ceraunus antibubastus*	Ceraunus blue Butterfly	Co.	T			X	X	X		X		X			2
LYCAENIDAE	*Icarcia icarioides fenderi*	Fender's Blue butterfly	Co.	En.			X	X								1
LYCAENIDAE	*Icarcia icarioides missionensis*	Mission blue butterfly	Co.	En.			X	X								1
LYCAENIDAE	*Leptotes cassius theonus*	Cassius blue Butterfly	Co.	T			X	X	X		X		X			2
LYCAENIDAE	*Lycaeides argyrognomon lotis*	Lotis Blue butterfly	Co.	En.				X	X							1
LYCAENIDAE	*Lycaeides melissa samuelis*	Karner Blue butterfly	Co.	En.				X					X			1
LYCAENIDAE	*Plebejus shasta charlestonensis*	Mount Charleston blue	Co.	En.			X	X	X			X	X			2
LYCAENIDAE	*Strymon acis bartrami*	Bartram's Hairstreak	Co.	En.	X	X		X			X	X	X		X	2
LYCAENIDAE TOTAL					**1**	**1**	**10**	**14**	**5**	**2**	**3**	**3**	**7**	**1**	**1**	
NYMPHALIDAE	*Anaea troglodyta floridalis*	Florida Leafwing Buttefly	Co.	En.				X				X				2
NYMPHALIDAE	*Boloria acrocnema*	Uncompahgre Fritillary	Co.	En.					X				X			1
NYMPHALIDAE	*Euphydryas editha bayensis*	Bay Checkerspot	Co.	T	X	X	X	X			X		X			1
NYMPHALIDAE	*Euphydryas editha quino*	Quino Checkerspot butterfly	Co.	En.				X		X	X	X				1
NYMPHALIDAE	*Neonympha mitchellii francisci*	Saint Francis' Stayr butterfly	Co.	En.				X					X		X	1
NYMPHALIDAE	*Neonympha mitchellii mitchellii*	Mitchell's Satyr Butterfly	Co.	En.			X	X					X	X		1
NYMPHALIDAE	*Speyeria callippe callippe*	Callipe Silverspot butterfly	Co.	En.			X	X		X				X		1

Table 1.1. (Continued) Federally Threatened (T) and Endangered (En.) butterflies currently listed by USFWS and the threats reported for each species. Habitat alteration is considered to be any direct impact on butterfly habitat by humans (e.g. Urban expansion, forestry, agriculture, mining, logging). Species whose distributions included both continents and islands are considered to be continental. Reference codes: 1. Xerces Society; 2. Federal Register document for respective species from USFWS Environmental Conservation Online System.

NYMPHALIDAE	*Speyeria zerene behrensii*	Behren's silverspot	Co.	En.			X	X		X		X		X		1
NYMPHALIDAE	*Speyeria zerene hippolyta*	Oregon Silverspot butterfly	Co.	T				X				X				1
NYMPHALIDAE	*Speyeria zerene myrtleae*	Myrtle's Silverspot butterfly	Co.	En.			X	X		X		X		X		1
NYMPHALIDAE	*Hypolimnas octocula marianensis*	Mariana Eight-Spot	Is.	En.		X		X	X	X					X	2
NYMPHALIDAE	*Vagrans egistina*	Mariana Wandering	Is.	En.		X			X	X					X	2
NYMPHALIDAE TOTAL					**1**	**3**	**5**	**10**	**3**	**6**	**2**	**5**	**4**	**4**	**3**	
PAPILIONIDAE	*Heraclides aristodemus ponceanus*	Schaus Swallowtail butterfly	Co.	En.				X	X				X	X		1
PAPILIONIDAE TOTAL					**0**	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**1**	**1**	**0**	
RIODINIDAE	*Apodemia mormo langei*	Lange's Metalmark butterfly	Co.	En.			X	X	X							1
RIODINIDAE TOTAL					**0**	**0**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	
GRAND TOTAL					**2**	**4**	**16**	**26**	**10**	**8**	**5**	**8**	**12**	**6**	**4**	

Primary Threats Identified

Lepidoptera are heavily impacted by numerous threats worldwide. A review of the scientific literature, agency documents, and species recovery plans revealed that threatened and endangered butterflies suffer impacts from a diversity of factors. The most frequently cited single category of factors was habitat alteration (79% of species), followed by invasive plants (26%) climate change (24%), grazing (23%), fires (17%), over-collecting (17%), suppression of natural fires (11%), pesticides (9%), introduced predator/parasite (9%), and disease (4%). In addition, other threats were cited for 26% of the species (Table 1.2). These other threats included habitat abandonment allowing other plants (native and non-native) to outcompete host plants, natural weather disasters (e.g. typhoons), and indirect impacts caused by population changes in other species (e.g. beavers). Many of these threats are summarized below, along with the most relevant examples of each.

Table 1.2. Butterflies of the IUCN Red List and USFWS- ECOS (Table 1.1) from the families: Hesperiidae, Lycaenidae, Nymphalidae, Papilionidae, Pieridae, and Riodinidae. Only species listed as "extinct", "critically endangered", "endangered", and "vulnerable" were included from the IUCN Red List. Species listed as "endangered" (N=23) by both USFWS- ECOS and IUCN were grouped together in the endangered category. Species listed by USFWS- ECOS as "threatened" (N= 5) were combined with species listed by IUCN as "vulnerable". Available information on each species' threat was compiled by family. For many species, multiple threats were cited. Habitat alteration is being considered to be any direct effect to a butterfly's habitat by humans (e.g. urban expansion, forestry, agriculture, logging, mining).

	Species	Extinct	Critically Endangered	Endangered	Vulnerable	Species w/ Threats Specified	Disease	Introduced Predator/Parasite	Invasive Plants	Habitat alteration	Climate change	Grazing	Fires	Suppression of Natural Fires	Over-Collecting	Pesticides	Other Threats
Continent	**143**	**3 (2%)**	**6 (4%)**	**45 (32%)**	**89 (62%)**	**59 (41%)**	**2 (3%)**	**2 (3%)**	**18 (31%)**	**49 (83%)**	**15 (25%)**	**14 (24%)**	**10 (17%)**	**8 (14%)**	**12 (20%)**	**6 (10%)**	**12 (20%)**
HESPERIIDAE	3 (2%)	0	0	1 (33%)	2 (67%)	2 (67%)	0	0	1 (50%)	1 (50%)	1 (50%)	0	1 (50%)	0	0	0	1 (50%)
LYCAENIDAE	104 (73%)	3 (3%)	4 (4%)	28 (27%)	69 (66%)	32 (31%)	1 (3%)	1 (3%)	11 (34%)	28 (88%)	9 (28%)	7 (22%)	4 (13%)	3 (10%)	7 (22%)	1 (3%)	7 (22%)
NYMPHALIDAE	24 (17%)	0	2 (8%)	14 (58%)	8 (33%)	20 (83%)	1 (5%)	1 (5%)	5 (25%)	16 (80%)	3 (15%)	6 (30%)	4 (20%)	5 (25%)	4 (20%)	4 (20%)	3 (15%)
PAPILIONIDAE	8 (6%)	0	0	1 (13%)	7 (88%)	1 (13%)	0	0	0	1 (100%)	1 (100%)	0	0	0	1 (100%)	1 (100%)	1 (100%)
PIERIDAE	3 (2%)	0	0	0	3 (100%)	3 (100%)	0	0	0	2 (67%)	0	1 (33%)	1 (33%)	0	0	0	0
RIODINIDAE	1 (1%)	0	0	1 (100%)	0	1 (100%)	0	0	1 (100%)	1 (100%)	1 (100%)	0	0	0	0	0	0
Islands	**75**	**1 (1%)**	**3 (4%)**	**28 (37%)**	**43 (57%)**	**11 (15%)**	**1 (9%)**	**4 (36%)**	**0**	**6 (55%)**	**2 (18%)**	**2 (18%)**	**2 (18%)**	**0**	**0**	**0**	**6 (55%)**
NYMPHALIDAE	40 (53%)	1 (3%)	1 (3%)	17 (43%)	21 (53%)	7 (18%)	0	2 (29%)	0	3 (43%)	2 (29%)	2 (29%)	2 (29%)	0	0	0	6 (86%)
PAPILIONIDAE	32 (43%)	0	1 (3%)	9 (28%)	22 (69%)	1 (3%)	0	0	0	1 (100%)	0	0	0	0	0	0	0
PIERIDAE	3 (4%)	0	1 (33%)	2 (67%)		3 (100%)	1 (33%)	2 (67%)	0	2 (67%)	0	0	0	0	0	0	0
Grand Total	**218**	**4 (2%)**	**9 (4%)**	**73 (34%)**	**132 (61%)**	**70 (32%)**	**3 (4%)**	**6 (9%)**	**18 (26%)**	**55 (79%)**	**17 (24%)**	**16 (23%)**	**12 (17%)**	**8 (11%)**	**12 (17%)**	**6 (9%)**	**18 (26%)**

Anthropogenic Habitat Alteration

In the context of this review, anthropogenic habitat alteration is considered to be any direct destruction or conversion of habitat by humans, as opposed to indirect habitat alterations such as those caused by climate change and invasive species. A common form of anthropogenic habitat alterations is urbanization, which is one of the leading anthropogenic threats to butterflies (Tables 1 & 2; Clark et al., 2007). Clearing of land for agriculture, logging and mining, and increased density of urban features such as roads, buildings, and mowed lawns, have been found to correspond with decreases in butterfly abundance, species richness, and diversity (Clark et al., 2007). According to a global study, urban land is expected to more than double from about 600,000 km^2 in 2000 to over 1,250,000 km^2 in 2030 (Fragkias & Seto, 2012). Much of this soon to be converted land may replace natural vegetation with highly disturbed unnatural environments which accelerate the establishment and spread of alien species. Because butterflies are host plant specialists, any change in vegetation or climate that leads to the reduction or loss of their host plants could dramatically reduce their populations (New, 2014; Clark et al., 2007).

A case in which urban expansion has severely impacted a butterfly species is the extinction of the Xerces blue, *Glaucopsyche xerces* Boisduval (Lycaenidae). Not much is known about the life history of this species, but its extinction in 1941 is primarily credited to the urban sprawl of San Francisco City six to eight decades prior, which resulted in the destruction of its native sand dune habitat and removal of native vegetation (Emmel & Emmel, 2014). Another species threatened by urbanization, but tentatively saved from extinction, is the Brenton Blue butterfly, *Orachrysops niobe* Trimen (Lycaenidae) in South Africa. The only known population was secluded to an area less than 1 km^2 known as Brenton-on-Sea, which was being threatened by housing construction (Henning et al., 2009). This led to a campaign known as the 'Brenton Blue Project' in the latter half of the 1990's (New, 2014), and in 2003, the area was proclaimed as a Special Nature Reserve known as 'Brenton Blue Butterfly Reserve', and the construction was halted, enabling the persistence of the butterfly (Edge, 2015).

Due to increasing human populations, agricultural landscapes worldwide are under increased pressure to provide food, feed, fiber, and fuel (Meehan et al., 2013). Several studies have been conducted to determine the effects that conversion of land to agriculture has on local butterflies. Monarch butterflies, *Danaus plexippus* L. (Nymphalidae), for example, are thought to be indirectly affected by the increase of glyphosate usage in corn and soybean fields throughout the Midwestern U.S. When the use of genetically modified glyphosate-resistant crops increased, milkweed host plants in agricultural lands became more scarce, and from 1999-2010, monarch egg production in the Midwest was reduced by 81% (Pleasants & Oberhauser, 2012). Also in the Midwestern U.S., a study found that as more acreage was converted to crop land, overall butterfly diversity decreased, and agricultural areas containing a low percentage of crop land, high percentage of woodland, and intermediate percentage of grassland and wetland, contained the highest butterfly species richness (Meehan et al., 2013). In another study, butterfly communities in undisturbed riparian habitats were compared with agricultural riparian habitats in the Walker River Basin (Nevada & California, USA). Although butterfly abundance was greater in the agricultural sites, the species present were geographically widespread and characteristic of disturbed habitats, suggesting that although riparian habitats created to support agriculture may support some native butterfly species, they are not good substitutes for undisturbed riparian habitats (Fleishman et al., 1999). A third study tested the effect of cultivated crops on the species richness and abundance of fruit-feeding butterflies in the mid-Zambezi valley of northern Zimbabwe (Tambara et al., 2013). The clearing of natural woodlands for cultivation purposes had a negative impact on butterfly diversity, and the more intensely the area was cultivated, the greater the negative impact (Tambara et al., 2013).

Logging and mining are also major threats to butterfly habitats. Like urbanization and agriculture, logging and mining directly impact butterfly habitats by clearing large areas of natural vegetation. In some cases, natural forests and vegetation are necessary for survival, even when these habitats do not include larval host plants. Every year, millions of monarch butterflies migrate to overwintering sites in forests of Mexico. However, in recent years scientists began noticing a drastic decline in monarch populations (Brower et al., 2002; Brower et al., 2009; Vidal et al., 2014). One major threat is the deforestation of their overwintering sites. Between 1971 and 1999, 44% of conserved forest was degraded, and the largest patch of high-quality forest was reduced by 79% (Brower et al., 2002). Between 2001 and 2012, 94% of 2179 ha used for overwintering was affected by illegal logging (Vidal et al., 2014). Monarchs use the forest as a "blanket and an umbrella" to prevent them from getting wet and freezing during the harsh winter months (Anderson & Brower, 1996). A study showed that butterflies with water on their body surfaces froze at higher temperatures compared to butterflies with no water on their bodies, providing evidence that dry refuges are important for survival. (Anderson & Brower, 1996). These studies further reinforce the fact that forest degradation can have a major impact on butterfly species and their habitat, even when larval host plants are not affected.

A review of the endangered *Kallima albofasciata* Moore (Nymphalidae), endemic to the Andaman Islands, speculated that British colonization and associated clearing of forests in the late 19th century may have adversely impacted the butterfly population within a century's time (Kamalanathan & Mohanraj, 2012). In the mid 1800's, shortly after the British annexed the islands, the butterfly was commonly found, but almost a century later the butterfly had become scarce and was rarely collected (Kamalanathan & Mohanraj, 2012). Although it is possible that invasive species, over-collecting, and other threats came along with colonization, this is not mentioned as a potential cause of decline.

Invasive Plants

Introduced plants have led to butterfly declines in some species. One example is the case of the endemic Richmond birdwing butterfly, *Ornithoptera richmondia* Gray (Papilionidae), in Australia. This species was once common but was reduced to about one-third of its former range by the early 1990's (New, 2014). Along with deforestation concerns, its decline is thought to be the result of an introduced vine that escaped from garden cultivation. The South American Dutchman's pipe vine, *Aristolochia littoralis* D. Parodi (Aristolochiaceae), is an attractive host to the female birdwings for oviposition, but the foliage is extremely toxic to the larvae, killing them shortly after they begin to feed (New, 2014; Sands & New, 2002). After being adopted as a flagship species in the 1990's, the Richmond birdwing butterfly became the focus of a recovery plan involving the public in 1996 (New, 2014). Since then, the removal of wild Dutchman's pipe vine and out-plantings of its native host, *Pararistolochia praevenosa* ((F. Muell) Michael J. Parsons (Aristolochiaceae)), have continued (Sands et al., 1997; Sands & New, 2013). This resulted in the reappearance of the butterfly in areas from which they had been extirpated for decades, potentially making it a 'rehabilitated species' (Sands & New, 2002).

Perhaps more commonly than the aforementioned situation, invasive plants affect butterflies by outcompeting or displacing their native host plant(s), and altering the native ecosystem and its resources. A study done on the island of Mauritius sought to determine the effects of invasive plants, mainly strawberry guava, *Psidium cattleianum* Sabine (Myrtaceae), on species richness and abundance of local butterfly populations. The study compared butterfly observations between weeded and non-weeded areas, and found that in weeded areas, nine native butterfly species were observed in comparison to non-weeded areas where there were only three native butterfly species. These findings suggested that alien plant invasions may have contributed to the extinction of some of the island's endemic butterfly species and may continue to do so by reducing the habitat quality that many of the native butterfly species rely on (Florens et al., 2010). A similar study done in the Southeastern U.S. found that areas where invasive Chinese privet, *Ligustrum sinense* Lour. (Oleaceae), was removed supported more than twice as many butterfly species and over four-times higher butterfly abundance than areas infested with the weed (Hanula & Horn, 2011). Another study sought to determine the important factors causing the decline of a native North American butterfly, *Pieris napi oleracea* Harries (Pieridae). Along with parasitism, the authors concluded that the decline of host plants caused by the rapid spread of garlic mustard, *Alliaria petiolate* ((M.Bieb.) Cavara & Grande (Brassicaceae)), is a major factor and will continue to be since past research has shown that once the weed establishes it becomes a permanent resident (Keeler et al., 2006).

Introduced Insect Parasitoids and Predators

There are numerous examples of introduced parasitoids having serious impacts on endemic butterflies and moths (Lozan et al., 2008). One is the sharp decline of the once widespread small tortoiseshell butterfly, *Aglais urticae* L. (Nymphalidae), in Northern Europe from 2003 to 2008. Researchers suggested that the decline was due to a newly introduced nymphalid parasitoid specialist, *Sturmia bella* Meigen (Diptera: Tachinidae) (Gripenberg et al., 2011). Field and lab studies demonstrated that *S. bella* was spreading throughout the U.K. It was reared from 26% of collected *A. urticae* larvae, parasitizing *A. urticae* more than any native parasitoid, and caterpillar survival was 25-48% lower when *S. bella* was present (Gripenberg et al., 2011). The authors concluded that although other factors may play a role in the decline of *A. urticae*, *S. bella* is a major threat (Gripenberg et al., 2011). The non-target effects of other biocontrol agents such as the polyphagous tachinid fly, *Compsilura concinnata* Meigen (Diptera: Tachinnidae), have been studied in recent years (New, 2014). This biocontrol agent was released widely throughout the eastern United States from 1906 to 1986 to control the invasive gypsy moth (Kellogg et al., 2003). Scientists studied the non-target effects of the tachinid by comparing survival of deployed 'sentinel larvae' of the native luna moth, *Actias luna* L. (Saturniidae), within and outside the current range of the fly (Kellogg et al., 2003). In the invaded area, the authors found that 78% of the parasitism of *A. luna* was caused by *C. concinnata* (Kellogg et al., 2003). Although a few other parasitoid species were reared from sentinel larvae, *C. concinnata* was the most common, supporting the notion that the introduced tachinid is a serious threat to native species such as *A. luna* and possibly others (Kellogg et al., 2003).

In addition to introduced parasitoids, introduced predators also play a role in declines of Lepidoptera. One of the most commonly introduced insect predators is the seven-spot ladybird beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae). In a study on the endangered Karner Blue butterfly, *Plebejus melissa samuelis* Nabokov (Lycaenidae), in Wisconsin and Minnesota, the authors observed the predation of two second instar caterpillars by one adult *C. septempunctata*, and author's speculated that ladybird beetles could potentially have severe impacts (Schellhorn et al., 2005). Ants also can have severe impacts on butterfly populations. The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Myrmicinae) has been documented to attack all immature stages (egg, larva, and chrysalis) of the endangered Schaus swallowtail, *Papilio aristodemus ponceanus* Schaus (Papilionidae), in Florida, causing concern for the remaining butterfly populations (Forys et al., 2001). The yellow crazy ant, *Anoplolepis gracilipes* Smith F. (Hymenoptera: Formicidae), was also found to impact a native butterfly species, *Vindula arsinoe* Cramer (Nymphalidae), in Australia. A study using sentinel larvae found that the invasive yellow crazy ant attacked at much higher rates than the native tree ant, *Oecophylla smaragdina* Fabricius (Hymenoptera: Formicidae) (Lach et al., 2016).

Vertebrate Predators

Birds have been reported to be a major predator of lepidopteran larvae by a number of sources (Holmes et al., 1979; Price & Clancy, 1986; Marquis & Whelan, 1994; Altegrim, 1989). In a study comparing bird and spider effects on tussock moth larvae in Maryland, the authors found larval size to be an important factor. Large larvae (10-15mm) were more susceptible to avian predation, whereas small larvae (<5mm) were possibly more susceptible to hunting spiders if they fell to the ground (Medina & Barbosa, 2002). A similar study conducted at the Poamoho Research Station on Oahu, Hawaii, sought to determine the direct effect of birds and spiders on naturally colonizing Lepidoptera larvae on *Brassica* L. plants. Using a variety of trials, they found that birds were the most important predators for lowering caterpillar densities (Hooks et al., 2003). Spiders were found to feed on Lepidoptera larvae, but they had less impact. A study using sentinel artificial larvae in Papua New Guinea under two different treatments (exposed and semi-concealed), found that along with ants, birds exerted the highest attack rates overall, and significantly higher attack rates on caterpillars that were in the open, in comparison to those concealed in leaf rolls (Tvardikova & Novotny, 2012). These studies provide further evidence that birds are a major predator of Lepidoptera larvae.

A reptilian predator causing impacts to island insects is the green anole, *Anolis carolinensis* Voigt (Polychrotidae), which has been introduced to many islands in tropical and subtropical regions. This alien species is the primary suspect linked to the decline and extinction of many of Ogasawara Island's endemic insect species (Sugiura, 2016; Karube, 2010). Because it is a diurnal generalist predator, a number of insects are preyed upon by this lizard, including butterflies like the endemic lycaenid, *Celastrina ogasawaraensis* Pryer (Sugiura, 2016). Other invasive species such as the bullfrog, cane toad, black rats, feral goats, and invasive flora have been suspected to cause impacts on the endemic insects of Ogasawara as well, but evidence shows that the green anole is the main threat (Karube, 2010). In other areas, such as Hawaii, these invasive species could also have profound effects. The Jackson's chameleon, *Trioceros jacksonii* Boulenger (Chamaeleonidae), green anole (*A. carolinensis*), and several species of toads and frogs are currently established in Hawaii (McKeown, 1996). These reptiles and amphibians along with others, could feed on immature and adult stages of Lepidoptera depending on their size, habitat, and flight pattern.

Wild Ungulates

Wild ungulates such as pigs, goats, sheep, and deer may impact host plant populations of butterfly species by feeding on a wide variety of native fauna, especially in island ecosystems, like Hawaii, where the flora have evolved with little need for defenses against mammalian herbivory, such as thorns and prickles (Wagner & Van Dresche, 2010; Denslow, 2003; Hess, 2016). Many native plant species in Hawaii are endangered or extinct due to the introduction of goats, sheep, and deer. For example, on Lanai Island, about 20% of the 345 native vascular plants have disappeared, and three species endemic to the island are thought to have gone extinct (Hobdy, 1993). Following great concern, government agencies have ongoing eradication and suppression programs and have fenced areas off across the state, in an effort to protect what native plant species remain. On Lanai, goats and sheep were successfully eradicated in the 1980's, but deer and mouflon remain in high numbers (Hess, 2016). Even in cases when ungulates do not directly impact host plants of native insects, the feeding damage caused to the flora may cause indirect ecosystem-modifying impacts such as severe erosion (Hobdy, 1993).

Fires

Fires have been found to cause direct mortality and long term population declines in various butterfly species (Powell et al., 2007; Swengel, 1995). A study done on the effects of fire on the regal fritillary, *Speyeria idalia* Drury (Nymphalidae) populations in northeastern Kansas prairie land found that recently burned areas contained fewer of these butterflies than unburned areas, and that populations took several years to rebound after a fire (Powell et al., 2007). A broader study based on surveys of a few dozen butterfly species in the upper Midwest U.S. found similar results. The author found that specialist butterflies declined sharply after fires, and continued to dwindle for 3-5 years or more (Swengel, 1995). In contrast, generalist species were more common in recently burned areas and less common in areas that had been unburned (Swengel, 1995). This study not only confirms that fires can heavily impact butterfly populations, but its effects can vary among species, specialists being impacted the most.

Islands vs. Continents

Due to their isolation, islands are often rich in resources and many contain endemic species that have evolved in the absence of strong competition, herbivory, parasitism and predation (Courchamp et al., 2003; Denslow, 2003; Paulay, 1994; Vitousek et al., 1987). Because of the absence of many native groups of plants and animals, alien species are often able to easily establish and become invasive, and are thus considered a greater threat on islands compared to continents (Corlett, 2009; Courchamp et al., 2003; Dulloo et al., 2002; Kueffer et al., 2010; Denslow, 2003; Gillespie & Roderick, 2002; Florens et al., 2010; Elton, 1958).

Islands in general are known to support a higher concentration of endemic insect species than continents, as well as be more susceptible to insect extinctions and declines (Gillespie & Roderick, 2002). For example, since the 1600's, 51 island insect species are documented to have gone extinct compared to only 10 from continents (Gillespie & Roderick, 2002). In the Hawaiian Islands alone, there are more instances of extinct arthropod species than in the entire continental U.S., and the number of species listed as endangered candidates is twice that of any continental state (Gillespie & Roderick, 2002). These extinctions and declines of native species cannot be solely attributed to one specific threat, but as mentioned in previous sections, urbanization and alien species are major conservation issues on islands. Some alien species have been purposely introduced (e.g. biocontrol agents, ornamental plants) but most have been transported accidentally by humans (Nishida, 2002). Many island insects have also evolved unique adaptations to island habitats, such as losing their dispersal abilities, which predisposes them to be vulnerable to alien species (Gillespie, 2007). For example, in Hawaii, 10 of the 11 naturally occurring alate insect orders include endemic species that evolved to become flightless, and in New Zealand, 94% of Lepidoptera have limited dispersal abilities (Gillespie & Roderick, 2002).

To further investigate the difference between islands and continents, I used the species records on the IUCN Red List database and USFWS-ECOS to compare threats between the two ecosystem types. Table 1.2 contains species listed on the IUCN Red List database as "vulnerable", "endangered", "critical", or "extinct" from five butterfly families: Hesperiidae, Lycaenidae, Nymphalidae, Papilionidae, and Pieridae. Species listed as "endangered" (N=23) and "threatened" (N=5) from USFWS- ECOS (Table 1.1) were also combined under the categories "endangered" and "vulnerable" respectively. Species were classified according to their geographic distribution (continental or island) and threats were tabulated when information was available. Threats to IUCN Red List species were obtained from information provided on the species associated webpage.

Detailed information on threats was only available for 32% of species listed as being of conservation concern, illustrating the fact that there have been few studies investigating the causes of Lepidoptera decline, despite the declines themselves often being clearly evident. Even fewer studies have been done on island butterflies; information on threats could be found for only 15% of island butterflies as compared to 41% of continental butterflies (Table 1.2). Additionally, although islands cover only 5.3% of Earth's land mass (Tershy et al., 2015), they contain are home to 38% of the endangered butterflies.

Although it is difficult to confidently assess differences in threats associated with the two types of land masses, the important threats to butterflies seem to be habitat alterations, grazing, fires, invasive plants, over-collecting, and climate change regardless of their location. However, a few differences were the more frequent citations of the effects of the suppression of natural fires, over-collecting, and invasive plants on continents. It may be that islands have a lower occurrence of natural fires, and over-collecting is not an issue because of strict laws and regulations like in Hawaii. It might also be that less research has been conducted on island butterflies, as evidenced by the fact that specific information on threats could be found for only a small proportion of island species. Interestingly, invasive flora was not recorded to be a major issue to butterflies on islands but was on continents, even though invasive species are generally accepted to be more problematic on islands. Hawaii alone has an estimated 927 alien plant species established, many of which are highly invasive (Gillespie, 2007). One reason that effects of invasive plants on island butterflies might be under-documented may be because it is difficult to quantify the effects invasive flora can have on a specific insect species, since these effects are often indirect. In many cases, invasive plants can cause indirect impacts to animal populations by altering the ecosystem over relatively long timescales (Kitayama & Mueller-Dombois, 1995; Mueller-Dombois, 1972). Another difference is that island butterflies seem to be more susceptible to introduced predators and parasites (36%) in comparison to continental species (3%). This may be the case since islands are typically much smaller in area. This not only limits the ability of butterflies to escape into refugia, but it also allows introduced predators and parasites to establish and quickly saturate the landscape. Also, island species have often evolved in the absence of many groups of predators (e.g. ants in Hawaii) and parasitoids. One recent example is the Mariana eight-spot butterfly, *Hypolimnas octocula mariannensis* Fruhstorfer (Nymphalidae), of the Mariana Islands, which was declared an Endangered species in 2015 (Federal Register, 2015). The main threats to this species are thought to be host plant declines by ungulate grazing, predation by ants, and parasitism by small wasps (Federal Register, 2015; USFWS, 2013). Other threats island butterflies may face in comparison to continents are: urban expansion may be a bigger threat because of the small amount of land, host plants on islands may be rarer if they require specific requirements to grow, and island butterflies may naturally have small populations, so any declines could reduce numbers below sustainable levels (Gillespie & Roderick, 2002; Gillespie, 2007).

Potential Threats to Hawaiian Lepidoptera

The current fauna of the Hawaiian Archipelago developed over a period of 30 million years and consists of eight main islands and a series of small atolls (Ziegler, 2002). Being that it is roughly 3,200 km from the nearest continent, it has been estimated that only one species of plant or animal colonized the islands every 35,000 years prior to the arrival of humans (Loope, 1998; Ziegler, 2002). Hawaii is known for its high endemism, and its native species have evolved without the presence of many impactful groups of animals like ants and ungulates (Krushelnycky et al., 2005; Hess, 2016). Because of this, Hawaiian flora and fauna face severe impacts by invasive species, including those introduced purposefully (Kaufman, 2010; Gillespie & Roderick, 2002). Miller and Eldredge (1996) estimated there to be 7,979 insects occurring in the islands, and 32% of these are nonindigenous.

Ants

Because no ants are believed to be native to Hawaii, their introductions cause detrimental impacts to many native species, including Lepidoptera (Krushelnycky et al., 2005). Nearly 60 species of ants have been introduced to the islands (Krushelnycky, 2015), ranging from sea level to 2700 m in elevation (Krushelnycky et al., 2005). Although only a small percentage of native Lepidoptera have been studied, past research has found a common trend; when ants are present, native Lepidoptera decrease in abundance (Cole et al., 1992; Krushelnycky & Gillespie, 2010). One recent study found that the abundance of native *Hyposmocoma* spp. Butler (Cosmopterigidae) and *Eupithecia orichloris* Meyrick (Geometridae) caterpillars were significantly impacted on trees with high ant densities compared to trees with low ant densities (Krushelnycky, 2015). Future studies should be conducted to investigate the impacts of specific ant species on native Lepidoptera.

Insect Parasitoids

Hawaii supports only two native butterfly species and about 1000 native moth species (Nishida, 2002). Because of this, the majority of work done on native Lepidoptera has been done on moth species. Several studies have investigated the impacts of purposely and accidentally introduced parasitoids on endemic moths. One study done on the endemic moth, *Udea stellata* Butler (Crambidae), found that non-native parasitoid species could impact endemic Lepidoptera (Kaufman, 2010). In the two-year study, 3,531 wild larvae were collected from eight sites on Oahu, Kauai, and Hawaii Island. Of the surviving larvae, 43% were found to be parasitized by seven alien species, and every larval instar (6 total instars) was attacked by at least one parasitoid species (Kaufman, 2010). This study found that accidentally introduced species, such as *Trathala flavoorbitalis* Cameron (Hymenoptera: Ichneumonidae), had a higher impact than purposefully introduced biological control agents such as *Meteorus laphygmae* Viereck (Hymenoptera: Braconidae) (Kaufman, 2010). Another study, conducted on Oahu and Maui, corroborated the findings on *U. stellata*, showing that endemic moths in the genus *Omiodes* Guenee (Crambidae) were also impacted by both purposefully and accidentally introduced parasitoids, but the severity of impacts depended on the habitat and species of *Omiodes* (King et al., 2010). Furthermore, a broader study done in Alaka'i Swamp, Kauai, found that parasitoid biocontrol agents caused more mortality in native moths than accidentally introduced parasitoids (Henneman & Memmott, 2001). Out of 2,112 wild caterpillars collected, 216 parasitoids emerged, and 83% of those were biological control agents, 14% accidentally introduced, and 3% native (Henneman & Memmott, 2001). The two most common parasitoids were biocontrol agents: *M. laphygmae* (reared from 12 caterpillar species) and *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) (reared from 9 caterpillar species) (Henneman & Memmott, 2001). This study highlighted the fact that many biocontrol agents previously released in Hawaii are generalists, and may affect endemic Lepidoptera.

A two-year conservation assessment of Hawaiian Lepidoptera in the late 1970's to early 1980's came to the conclusion that the endangerment and extinction of Hawaii's native insects are primarily due to: habitat destruction and alteration, feral mammals and fire, introduction of polyphagous parasites and predators, destruction of certain host plants, exotic flora naturalization and lack of effective quarantine for in-bound commercial activity (Gagne, 1982). Although this study was based primarily on specimen records and anecdotal evidence, it provides insight into what threats are present in the state and further confirms that the threats present in Hawaii (except invasive flora) generally mirror those found on other islands (Table 1.2). Although invasive plants were not reported as a threat towards butterflies on other islands, they were reported as a threat to continental species, and should still be regarded as a high level threat in Hawaii considering the number of invasive plants (927 species) (Gillespie, 2007) that have already been introduced, and their displacement of many native plants that serve as hosts for native Lepidoptera. Sadly, however, studies conducted on Hawaiian Lepidoptera, particularly butterflies, have been scarce, and there is little comprehensive information on causes of decline of Hawaiian Lepidoptera.

Possible Causes of Decline of the Kamehameha butterfly

The Kamehameha butterfly, *Vanessa tameamea*, can be found throughout the state however it has become increasingly rare. Anecdotal evidence of declines in range and abundance were noted by the 1980s (Tabashnik et al., 1992), and the butterfly’s range has been reduced even further since then (Haines, 2014). The above review of factors affecting butterfly populations worldwide, might provide insight into why the Kamehameha butterfly is declining.

Many threats documented in other butterflies, such as fire (intentional or non-intentional), pesticides, and logging and mining industries, are unlikely to be current threats to the Kamehameha butterfly. In Hawaii, wildfires are not a frequent occurrence, and generally occur in dry areas outside of the Kamehameha butterfly's natural range since its host plants tend to be in wet regions. Hawaii does have an active volcano that causes changes in air quality and may be burning parts of butterfly habitat, but volcanic activity is limited on Hawaii Island, whereas the butterfly appears to be declining throughout the state, particularly on Oahu. Pesticides are mainly used in agricultural areas rather than the natural forest where host plants occur, and Hawaii does not have a large logging or mining industry.

Climate change may be a long-term threat, and is generally predicted to cause upward shifts in the range of Hawaiian species, towards higher elevations (Loope & Giambelluca, 1998). Little research has been done into the direct impacts of climate change on Hawaiian insects, and it is difficult to separate the effects of a warming climate from the effects of spreading invasive species, which are generally most prevalent in the lowlands, pushing native species into higher elevations (Cuddihy & Stone, 1990; Krushelnycky et al., 2005). Invasive plants are a major problem in Hawaii, and their impacts are not independent of climate change, since climate change may facilitate their spread to higher elevations (Loope & Giambelluca, 1998). Much of Hawaii's forests are being invaded by species such as strawberry guava, smothering native plants (Benitez et al., 2012). These invasive plants also provide habitat for other non-native species and cause fragmentation of natural habitats and forest (Benitez et al., 2012; Vargas et al., 1993).

The primary host plant for the Kamehameha butterfly, māmaki, *Pipturus albidus* ((Hook. & Arn.) A. Gray ex. H. Mann) (Rosales: Urticaceae), was not observed to be a particularly rare plant in native forests (Tabashnik et al., 1992). However, its range could be reduced due to invasive plants, as was found for other native plant species (Asner et al., 2008; Cox, 1999; LeQuire, 2009; Minden et al., 2010). On Oahu, māmaki was observed to be abundant in areas such as Tantalus and Kuliouou Valley, but the butterfly no longer occurs there, suggesting that it is limited by factors other than host plant abundance. A study of Kamehameha butterfly populations in the Tantalus area of Oahu and the Volcano area of Hawaii Island in the mid-1980s found that butterflies did not appear to be limited by larval food resources, and authors speculated that predators or parasitoids might be important factors (Tabashnik et al., 1992).

Invasive parasitoids and predators are known to impact Lepidoptera in Hawaii (Kaufman & Wright, 2009, 2010, 2011; King et al., 2010; King & Rubinoff, 2008; Gagne & Howarth, 1985; Funasaki et al., 1988; Gagne, 1982; Henneman & Memmott, 2001; Asquith & Miramontes, 2001). Some species that may be affecting *V. tameamea* larvae are: parasitoids (both native and nonnative), ants (all nonnative), lady-bird beetle larvae or adults (nonnative), lacewing larvae (native and nonnative), spiders (native and nonnative), and birds (native and nonnative). A suite of parasitoids have been reared from Kamehameha butterfly (Leeper, 2014; Swezey, 1912, 1913, 1915, 1922, 1927, 1929, 1931, 1934), but their population impacts have never been studied. It is also unclear what effect predators have on Kamehameha butterfly larvae.

Research Aims

Evaluate the impact of predators and parasitoids on eggs and larvae of the Kamehameha butterfly on Oahu

Objective 1. Develop a method for rearing the butterfly (Chapter 2).

In order to conduct the controlled exposure trials, large numbers of eggs and larvae will have to be produced in captivity. A sound method for rearing this butterfly has not been developed.

Objective 2. Evaluate the role of and organisms predating or parasitizing butterfly eggs and larvae (Chapter 3).

Sentinel larvae will be deployed at four sites on Oahu in various controlled-exposure treatments to assess the impact of birds, ants, and parasitoids. Sentinel eggs will be deployed at the same four sites with no controlled-exposure treatment. Ants will be surveyed using baited vials at each site.

Objective 3. Develop predictive simulations of survivorship of Kamehameha butterfly using the data generated from the controlled-exposure trials (Chapter 3)

The ultimate goal of this project is to gain an understanding of what has caused declines of this iconic insect, and what can be done to conserve it.

CHAPTER 2

LIFE HISTORY AND CAPTIVE REARING OF THE KAMEHAMEHA BUTTERFLY (*VANESSA TAMEAMEA*)

Introduction

This study aimed to increase the understanding of the basic biology of the Kamehameha butterfly and to develop a successful rearing methodology. In order to study parasitism and predation of the Kamehameha butterfly (*V. tameamea*) (see Chapter 3), it was necessary to first develop protocols to rearing them in a laboratory setting. Prior to this project, there was no information about how to establish a multigenerational colony. A previous attempt was made to rear caterpillars of this species, but the author had little success, commenting that they "proved delicate and temperamental", often succumbing to disease (Williams, 1928). Here the establishment of two colonies of the Kamehameha butterfly in a laboratory setting, and successful rearing for more than 15 generations in captivity are described. These methods will be useful for future research or reintroduction efforts for this species or similar species.

Because there was very little information in the scientific literature about the reproductive biology of *V. tameamea*, information on the biology of a closest known relative was used as a starting point for developing protocols. According to a recent phylogenetic study (Wahlberg & Rubinoff, 2011), the sister species to *V. tameamea* is the red admiral *V. atalanta* L. (Nymphalidae), a species native to North Africa and Eurasia that can also be found worldwide in temperate regions (Scott, 1986; Bryant et al., 1997). Like *V. tameamea*, *V. atalanta* larvae transition through five larval instars, and feed on herbs and shrubs in the Urticaceae family on which they form characteristic leaf shelters using silk (Scott, 1986; Wagner, 2005). Wing patterns are similar between the two species, each having red-orange, white and black variations on the upper surface and brown greenish tinged patterns on the underside. *V. atalanta* was introduced to Hawaii sometime prior to the 1890s, and utilizes some of the same host plants as *V. tameamea*, including māmaki (*P. albidus*).

Although it is not as easy to rear as other *Vanessa* spp. Fabricius (Nymphalidae), such as the painted lady, *Vanessa cardui* L. (Nymphalidae), methods for rearing *V. atalanta* for multiple generations in an indoor setting were developed by Dimock (1984). Eggs were initially obtained from wild-caught females, and larvae were reared gregariously in plastic boxes with moist tissue paper to promote humidity, and were fed foliage of *Urtica dioica* L. (Rosales: Urticaceae), as needed. Adults were raised in a flight cage next to a window, and were provided honey water solution for sustenance. The author noted a few important factors when rearing this species. He found that the air needed to be free of chemicals and insecticides, as all stages were susceptible to these contaminants. Humidity level in the rearing chamber was also important. High humidity aids larval growth and keeps foliage fresh, but also promotes disease, thus quick removal and separation of sickly or dead larvae was necessary. Finally, to encourage mating, ample cage space and proper location next to a window was essential since the butterflies preferred to mate during or after sundown (Dimock, 1984). To extend the mating hours, a flood light above the cage was successfully used to mimic sunlight. After mating, butterflies laid eggs readily on cuttings of *U. dioica*. The methodology developed for *V. atalanta* (Dimock, 1984) was very useful in developing the rearing protocols described in this chapter.

Laboratory Rearing Methods

Colonies of *V. tameamea* were initiated with wild caught females collected by netting while they visited sap fluxes to feed or host plants to oviposit from various sites around the Island of Oahu. Two colonies were established based on collections from the two main mountain ranges on Oahu Island: the Koolau and Waianae Ranges. Previous genetic research on the Kamehameha butterfly has shown that some populations on different islands are isolated from one another (W. Haines unpublished data). Although these studies found no evidence of isolation within islands, butterflies collected for colony establishment were kept separate by their respective mountain range to prevent unintended genetic exchange between populations. The Waianae colony was founded using wild females collected from Waianae Valley, and was later augmented with females from Palikea Trail and Kahanahaiki Gulch. The Koolau colony was founded using wild females collected from Kahana Valley State Park, and was not augmented with additional wild stock; although additional females were collected from Kahana Valley during the course of the study, obtaining eggs from them was unsuccessful.

After collection, the female butterflies were placed in glassine envelopes and kept in a dark cooler to keep them inactive during transport. They were taken back to the laboratory and placed individually in mesh cages (60.5 cm x 35 cm x 35 cm) where they were assigned a unique identification number so that their offspring could be tracked. The butterflies were allowed to lay eggs on a potted māmaki plant that was placed in the cage for oviposition. Although it is not possible to tell if a female has already mated upon collection, the majority of those collected laid fertile eggs. The butterflies were also provided with mashed fruit (mainly banana) along with a sugar and electrolyte solution soaked on a paper towel for food. The sugar and electrolyte solution included 94% energy drink, (Gatorade company "fruit punch" flavor, because it contained only natural flavors), 5.5% simple syrup (50% table sugar, 50% water), and 0.5% soy sauce (Kikoman company). Cages were misted with tap water several times each day to maintain humidity and hydration. Every day, the māmaki plants and cage walls were checked, and any eggs were removed, counted, and placed into covered 2 oz plastic cups labeled with the date, number of eggs, and the female ID to track the number of eggs laid by each female.

As the eggs hatched, each caterpillar was placed in an individual 2 oz plastic cup using a soft, fine paintbrush. Caterpillars were labeled with the female ID and mountain range, and a piece of māmaki leaf (about 2cm x 2cm) was provided as food. Cups containing caterpillars were stacked in loosely covered plastic bins with a moist paper towel to maintain humidity. When cups were not placed in bins, they often dried out quickly and required new leaves daily. The plastic bins containing the caterpillars were kept in the laboratory where the temperature was about 22 - 23°C. In the third generation, survival of individual caterpillars (N=442) from the Koolau colony was tracked, and 74% of caterpillars survived to pupation. Every one to two days, depending on the larval stage, cups were cleaned and fresh māmaki foliage was added.

Leaves used for feeding were harvested from potted māmaki grown in a greenhouse (University of Hawaii at Manoa, Gilmore Hall), and from wild plants at Lyon Arboretum and other sites where caterpillars were deployed (see Chapter 3). To maintain freshness, leaves were kept in sealed zippered bags and stored in environmental chambers at 14°C where they would last for up to seven days. Leaves kept in a conventional refrigerator (~4°C) seemed to last poorly in the colder temperatures, turning brown more rapidly. Caterpillars that could not be fed because of lack of food or time were stored in environmental chambers at 10°C for no more than 24 hours to slow their metabolism until food could be provided.

When larvae reached their final instar they were placed individually in larger 9 oz plastic cups due to their increased food consumption, and the space needed for successful pupation and eclosion. Caterpillars usually pupated on the lid of the container. The date of pupation was recorded on each cup, so dates of adult emergence could be measured. Using key physical features found on the chrysalis of other butterfly species, the sex of the pupae was attempted to be determined, but was unsuccessful for this species.

When adults eclosed, they were placed into large flight cages for mating. Cages were labeled and kept separate by mountain range so breeding between populations could not occur. At times, more than 100 butterflies were in each cage. As in the smaller oviposition cages containing wild females, the larger cages were provisioned with mashed fruit and electrolyte solution in feeder trays. Also in the cage were potted māmaki or other plants to provide a more natural setting. Cages were sprayed with water several times a day to maintain hydration of the paper towels, fruit, and butterflies. Adults survived for about a month in the laboratory, which is similar to what was found in a mark-recapture study done on Tantalus Mountain, Oahu, in which the longest-lived individual survived 35 days (Tabashnik et al., 1992).

Mating Preferences

From past records, it is known that the related *V. atalanta* mates during sundown (Dimock, 1984). Assuming the Kamehameha butterfly had similar mating behaviors and cues, various locations and cage sizes were attempted to provide natural lighting and achieve successful mating. The first attempts involved putting the butterflies in a large 10-person tent set up first on the roof of Gilmore Hall and later in an outdoor field plot at Magoon Laboratory in Manoa. Butterflies were released in the cage during the cooler afternoon hours and left overnight. In the morning, the butterflies would be transferred to smaller cages and moved back into the laboratory to avoid the heat of the oncoming day. Behavior was monitored in the early evening from just before sundown until after dark. To address concerns of over-handling during the first attempt, the second attempt involved placing the butterflies in large, heavy metal-framed cages (155 cm tall, 94 cm deep, and 98 cm wide) designed for fruit fly rearing. These cages had wheels, allowing to transport the cages to the roof before sundown and back indoors after dark. The final attempt was the simplest, consisting of large cages measuring 167 cm tall, 83 cm deep, and either 120 cm (Waianae) or 170 cm (Koolau) wide. Cages were constructed of PVC piping draped with black mosquito netting, and were placed next to a window in a temperature-controlled rearing room (24°C). All cage types were checked nightly for mating, and mated pairs were carefully captured in 9 oz cups. The following morning, pairs would be found separated. Male butterflies were released back into the mating cage so they could potentially mate again, while females were placed in smaller oviposition cages (see above) with potted māmaki plants.

The first attempt, which involved moving butterflies into a large outdoor tent during the late afternoon, was unsuccessful; no mating was observed after about two weeks, although evening courtship and/or territorial behavior often occurred. The second method, where butterflies were placed in transportable cages and moved outdoors in the late afternoon, would occasionally result in mating, and sometimes mating would occur indoors during early to mid-afternoon hours. Although mating occurred, it was not as reliable as the third and final method, which involved keeping butterflies in large mating cages next to a window and dimming or turning overhead lights off in the late afternoon or early evening. However, like with *V. atalanta* (Dimock, 1984) mating hours can be extended using lights. In this case, one bay of fluorescent lights was left on for one to two hours after dark, and mating often continued well after dusk. Over the course of 2 years, 15 generations were produced using these rearing methods. The number of eggs laid varied with female. 14 females were monitored from the second and third generation, and the number of eggs laid ranged from 20 to 181. This fecundity is lower than that documented by Dimock (1984) for *V. atalanta* females, which laid more than 300 eggs.

Mating Behavior

Butterflies generally did not begin courtship behavior until 3-5 days after eclosion, similar to what was previously documented for *V. atalanta* (Dimock, 1984). In all the cage types tested, as sundown approached, the activity of butterflies often increased notably, and they could be seen fluttering and chasing one another throughout the cages, presumably as part of courtship, though this behavior may also have been territorial. Typically, males initiated contact with the females, but some females were also observed initiating contact with males and in many cases butterflies of the same sex chased one another. To initiate mating, males would land next to another butterfly, usually of the opposite sex, and approach it while bending their abdomen at a right angle towards the recipient in an attempt to make contact. Often, more than one attempt was made before a successful connection occurred. If a female was not receptive, males tended to retry with another female. Though it was not recorded, occasionally, two males were observed copulating with the same female simultaneously. Courtship and mating activity varied dramatically from night to night. On some nights, butterflies were relatively inactive and there was no mating, and on other nights butterflies were extremely active, and there were many (up to 32) mating pairs in a cage. It is suspected that mating activity may be influenced by a number of factors including the number of available butterflies, their health and age, and weather conditions. It is uncertain to what degree pheromones are involved in the mating process. Once copulated, pairs rapidly became inactive and unresponsive, usually perching on the side or ceiling of the cage, and were easily captured without disrupting them. Mating pairs remained copulated well into the night, apparently separating at dawn.

General Life History Observations

Some information on the life cycle of *V. tameamea* is available from previous studies (Swezey, 1912; Williams, 1928; Zimmerman, 1958, Tabashnik et al., 1992). Like all Lepidoptera, the Kamehameha butterfly goes through four major life stages: egg, larva, pupa, and adult. Eggs are typically laid singly on both the upper and underside of host plant leaves. Eggs are typically light brown in color until a day or two before they are ready to hatch. Just before hatching, the black head capsule of the caterpillar has been observed, and the larva can be seen moving in the egg when viewed under a dissecting microscope.

Larvae are host specific, feeding only on a few native trees and shrubs in the nettle family (Urticaceae) (Zimmerman, 1958), like other related species of *Vanessa* (Wahlberg & Rubinoff, 2011). Māmaki is the most common host plant, but other hosts include opuhe, *Urera* spp. ((Hook. & Arn.) Wedd. (Urticaceae)), akolea, *Boehmeria grandis* ((Hook. & Arn.) A. Heller (Urticaceae)), and olona, *Touchardia latifolia* Gaudich. (Urticaceae) (Williams, 1928). Larvae of *V. tameamea* typically go through five instars before pupation (Leeper, 1975; Tabashnik et al., 1992), like most butterfly caterpillars (Field, 2013). A distinctive characteristic of instars one through four, is their leaf folding behavior (Swezey, 1912; Williams, 1928). These earlier instars cut semi-circular flaps in the leaves of their host plant and fold them over themselves, presumably for protection from predators or parasitoids, a behavior similar to that of related *Vanessa* spp. (Scott, 1986; Wagner, 2005).

Newly hatched caterpillars are often 2-3 mm long, and sparsely covered in long hairs (Fig. 2.1A). Upon hatching, they are grey in color and take on a green color after feeding (Fig. 2.1A). Typically, instars two through four are dark purple or black, mottled with light green or yellow, with soft spines protruding from thoracic and abdominal segments (Fig. 2.1B-D). Fourth instar caterpillars are often lighter in color than previous instars, and can be predominantly light green or purple (Fig. 2.1D). Head capsules of instars two through four are black or brown with short, blunt spines. The fifth instar changes drastically, and is typically bright green with black and red branched spines, with a lateral yellow stripe (Fig 2.1E). They also have a bright green heart-shaped head capsule with numerous short, white-tipped spines, and a reddish medial triangular stripe (Gorelick & Wielgus, 1968). A dark color morph, in which the fifth instar caterpillar is a purplish brown in color, has been recorded (Williams, 1928), but this morph did not occur in the laboratory colony, nor was it ever found among wild-collected larvae on Oahu. This brown morph has been collected on the islands of Hawaii, Maui, and Kauai (W. Haines personal observation). During its final instar, the caterpillar experiences the most growth, and is usually 4-5 cm in length before it pupates. Prior to pupation, the larva produces a thick pad of silk and hangs upside down from its rear prolegs in a “J” shape for about a day as a prepupa. The prepupa proceeds to molt its outer skin to metamorphose into a chrysalis where it remains for 10 to 11 days at laboratory temperature (22-23°C). The chrysalis of this species is variable in color, ranging from very light brown to dark brown to copper colored, with shiny metallic specks.

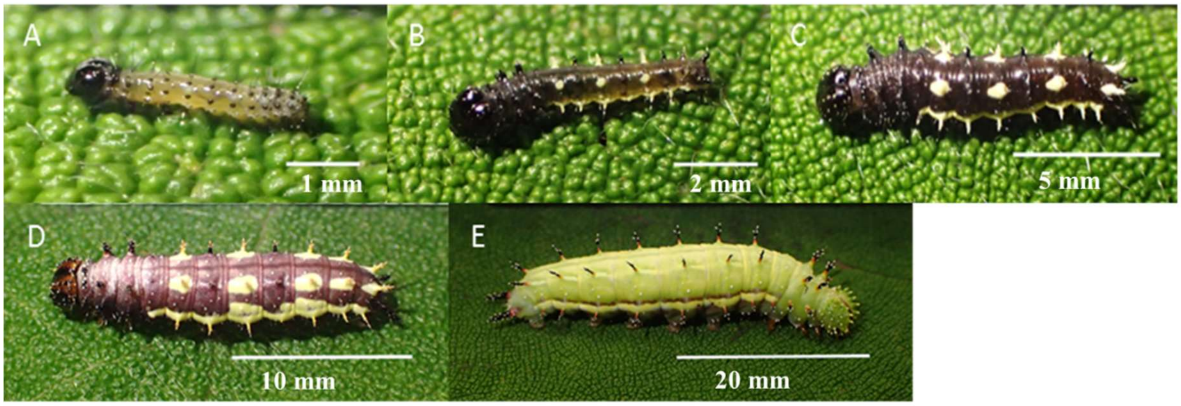

Figure 2.1. Typical color patterns and approximate lengths (mm) of *V. tameamea* caterpillar instars. A. 1st instar; B. 2nd instar; C. 3rd instar; D. 4th instar; E. 5th instar.

The wings of adult butterflies are black with orange patterns on the upper surface, and mottled brown on the lower surface, often with a greenish tinge. The black regions near the tips of the forewings have several white or orange dots that differ between sexes. In males, at least some of these dots are orange, whereas in females all of the dots are white (Williams, 1928; Fig. 2.2). Adults are known to congregate and feed on fermenting sap fluxes of koa, *Acacia koa* Gray (Fabaceae), as well as other native and non-native trees (Williams, 1928; Leeper, 1975), a behavior shared with *V. atalanta* (Zimmerman, 1958; Scott, 1986). Due to scant research on pollination in Hawaii, little is known about the importance of this species as a pollinator in the native ecosystem. However, the Kamehameha butterfly has been observed visiting the flowers of many native plants, including endangered species in the hibiscus family such as *Hibiscadelphus giffardianus* Rock (Malvaceae) (Pratt et al., 2010). Butterflies around the world are known to be important pollinators (Courtney et al., 1981; Goldblatt & Manning, 2002; Kremen et al., 2007; Weiss & Lamont, 1997) and possible indicators for tracking diversity in threatened ecosystems (Ehrlich & Ehrlich, 1981), suggesting that the Kamehameha butterfly may play an important role in Hawaiian forests, especially since it is one of only two native butterflies (Zimmerman, 1958; Williams, 1928). Because of its potential ecological importance, and the fact that it is a very charismatic insect, there is interest in captive propagation of this species. It is hoped that the methods described here will benefit conservation efforts, though it is important to note that if not conducted carefully, mass rearing and release of this species or any insect, can potentially cause more harm than good (Altizer et al., 2015; Frankham, 2008). Any captive rearing efforts should be conducted with great care to avoid potential harm caused by inbreeding, pathogens, or unnatural mixing of population genes from different islands or regions. In addition, permits for rearing this species or any other native species in captivity is required by the Hawaii Department of Land and Natural Resources.

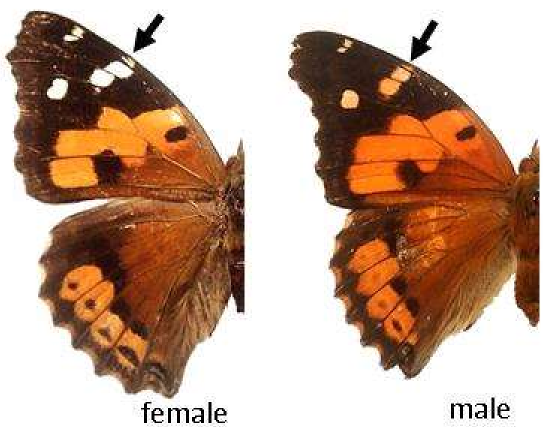

Figure 2.2. Wing patterns of female (left) and male (right) *V. tameamea* butterflies

Head Capsule Widths

A small trial to determine the head capsule widths of each instar was conducted on a subset of caterpillars (Table 2.1). Caterpillars were photographed after each molt next to a scalebar, and measurements of head capsules were taken in millimeters using Image J 1.48v (Rasband, 2014). Although the sample size used in this study was relatively small (n= <15), no overlap of head capsule widths was found between instars, suggesting that this measurement can be used to assign caterpillars to instar. The average width ranged from 0.74 mm (first instars) to 4.33 mm (fifth instars). It should be noted, however, that an additional instar (6 instars total) was occasionally observed in some of the colony caterpillars, and the head capsules of these caterpillars likely differed in head capsule width though it was not quantitatively measured.

Table 2.1. Head capsule width (HCW) in millimeters (mm) with standard error (±) by larval instar.

Instar	Mean HCW ± S.E. (mm)	Min HCW (mm)	Max HCW (mm)
1 (N=8)	0.74 ± 0.02	0.70	0.82
2 (N=12)	1.08 ± 0.017	0.93	1.16
3 (N=15)	1.70 ± 0.018	1.58	1.80
4 (N=12)	2.81 ± 0.049	2.54	3.12
5 (N=11)	4.33 ± 0.067	4.09	4.76

Effect of Temperature on Development Time

When rearing this species, temperature played an important factor for the growth of immature stages. Since colonies were primarily being reared for use in field experiments at various elevations, laboratory trials were conducted representing a range of temperatures likely to be observed. Quadratic trendlines (polynomial lines, order 2) were found to fit the data the best (Table 2.3), and were used to estimate the duration of each instar at each location. This was an important factor for calculating the risk of parasitism and predation at each site (see Chapter 3). Following the rearing methods described above, a subset of randomly selected eggs (N= 10 per temperature) and caterpillars (N= 20, 20, 20, 30, 20) were raised to adult eclosion in temperature controlled chambers (VWR diurnal growth chamber, Model 2015) set to 16, 18, 20, 22, and 24°C, respectively (Table 2.2). Eggs and caterpillars were separated into individual containers and assigned an ID number. Caterpillars were then checked daily for mortality and given adequate food, and the dates of molting, pupation, and adult eclosion were recorded. 30 larvae were used for the 22°C trial because a separate study comparing larval development on artificial diet (N=20) was being conducted side by side in the same chamber, and half of these larvae were raised on māmaki. Since the conditions were the same, the data from these 10 caterpillars were combined with the 20 caterpillars used for the temperature trials.

Table 2.2. Comparison between temperature and individual stage growth with standard errors. Sample sizes for each stage include only individuals that successfully completed that stage.

	Species	Extinct	Critically Endangered	Endangered	Vulnerable	Species w/ Threats Specified	Disease	Introduced Predator/Parasite	Invasive Plants	Habitat alteration	Climate change	Grazing	Fires	Suppression of Natural Fires	Over-Collecting	Pesticides	Other Threats
Continent	**143**	**3 (2%)**	**6 (4%)**	**45 (32%)**	**89 (62%)**	**59 (41%)**	**2 (3%)**	**2 (3%)**	**18 (31%)**	**49 (83%)**	**15 (25%)**	**14 (24%)**	**10 (17%)**	**8 (14%)**	**12 (20%)**	**6 (10%)**	**12 (20%)**
HESPERIIDAE	3 (2%)	0	0	1 (33%)	2 (67%)	2 (67%)	0	0	1 (50%)	1 (50%)	1 (50%)	0	1 (50%)	0	0	0	1 (50%)
LYCAENIDAE	104 (73%)	3 (3%)	4 (4%)	28 (27%)	69 (66%)	32 (31%)	1 (3%)	1 (3%)	11 (34%)	28 (88%)	9 (28%)	7 (22%)	4 (13%)	3 (10%)	7 (22%)	1 (3%)	7 (22%)
NYMPHALIDAE	24 (17%)	0	2 (8%)	14 (58%)	8 (33%)	20 (83%)	1 (5%)	1 (5%)	5 (25%)	16 (80%)	3 (15%)	6 (30%)	4 (20%)	5 (25%)	4 (20%)	4 (20%)	3 (15%)
PAPILIONIDAE	8 (6%)	0	0	1 (13%)	7 (88%)	1 (13%)	0	0	0	1 (100%)	1 (100%)	0	0	0	1 (100%)	1 (100%)	1 (100%)
PIERIDAE	3 (2%)	0	0	0	3 (100%)	3 (100%)	0	0	0	2 (67%)	0	1 (33%)	1 (33%)	0	0	0	0
RIODINIDAE	1 (1%)	0	0	1 (100%)	0	1 (100%)	0	0	1 (100%)	1 (100%)	1 (100%)	0	0	0	0	0	0
Islands	**75**	**1 (1%)**	**3 (4%)**	**28 (37%)**	**43 (57%)**	**11 (15%)**	**1 (9%)**	**4 (36%)**	**0**	**6 (55%)**	**2 (18%)**	**2 (18%)**	**2 (18%)**	**0**	**0**	**0**	**6 (55%)**
NYMPHALIDAE	40 (53%)	1 (3%)	1 (3%)	17 (43%)	21 (53%)	7 (18%)	0	2 (29%)	0	3 (43%)	2 (29%)	2 (29%)	2 (29%)	0	0	0	6 (86%)
PAPILIONIDAE	32 (43%)	0	1 (3%)	9 (28%)	22 (69%)	1 (3%)	0	0	0	1 (100%)	0	0	0	0	0	0	0
PIERIDAE	3 (4%)	0	1 (33%)	2 (67%)		3 (100%)	1 (33%)	2 (67%)	0	2 (67%)	0	0	0	0	0	0	0
Grand Total	**218**	**4 (2%)**	**9 (4%)**	**73 (34%)**	**132 (61%)**	**70 (32%)**	**3 (4%)**	**6 (9%)**	**18 (26%)**	**55 (79%)**	**17 (24%)**	**16 (23%)**	**12 (17%)**	**8 (11%)**	**12 (17%)**	**6 (9%)**	**18 (26%)**

Table 2.3. Quadratic trendline equations used to estimate duration of each developmental stage (see Chapter 3). y= duration (d), and x= temperature (°C).

Stage	Quadratic Trendline	R^2
Egg	$y = -0.0018x^2 - 0.7136x + 22.96$	0.987
1st	$y = 0.0179x^2 - 1.1543x + 20.64$	0.976
2nd	$y = 0.0768x^2 - 3.7264x + 49.22$	0.970
3rd	$y = 0.025x^2 - 1.57x + 27.04$	0.987
4th	$y = 0.0482x^2 - 2.6536x + 40.28$	0.949
5th	$y = 0.0821x^2 - 4.1757x + 62.02$	0.987
Pupa	$y = 0.1679x^2 - 9.0743x + 130.26$	0.975

The relationship between life cycle duration and temperature remained very consistent, indicated by the very low standard errors (Table 2.1, Fig. 2.3). As predicted, it was found that at lower temperatures, body processes and metabolism slowed down for all immature stages, while as temperatures increased, developmental rates also increased (Table 2.2, Figs. 2.3, 2.4). For instance, with an increase of 8°C (16°C to 24°C) the duration of the immature life cycle was halved from 99.0 days to 44.2 days. Furthermore, as temperature increased, growth rates began leveling off suggesting that the immature stages can only develop so fast before they reach a metabolic or developmental limit (Fig. 2.3). At 24°C, mortality was found to be highest in comparison to the cooler temperatures (personal observation) further suggesting that there is a temperature limit for caterpillars.

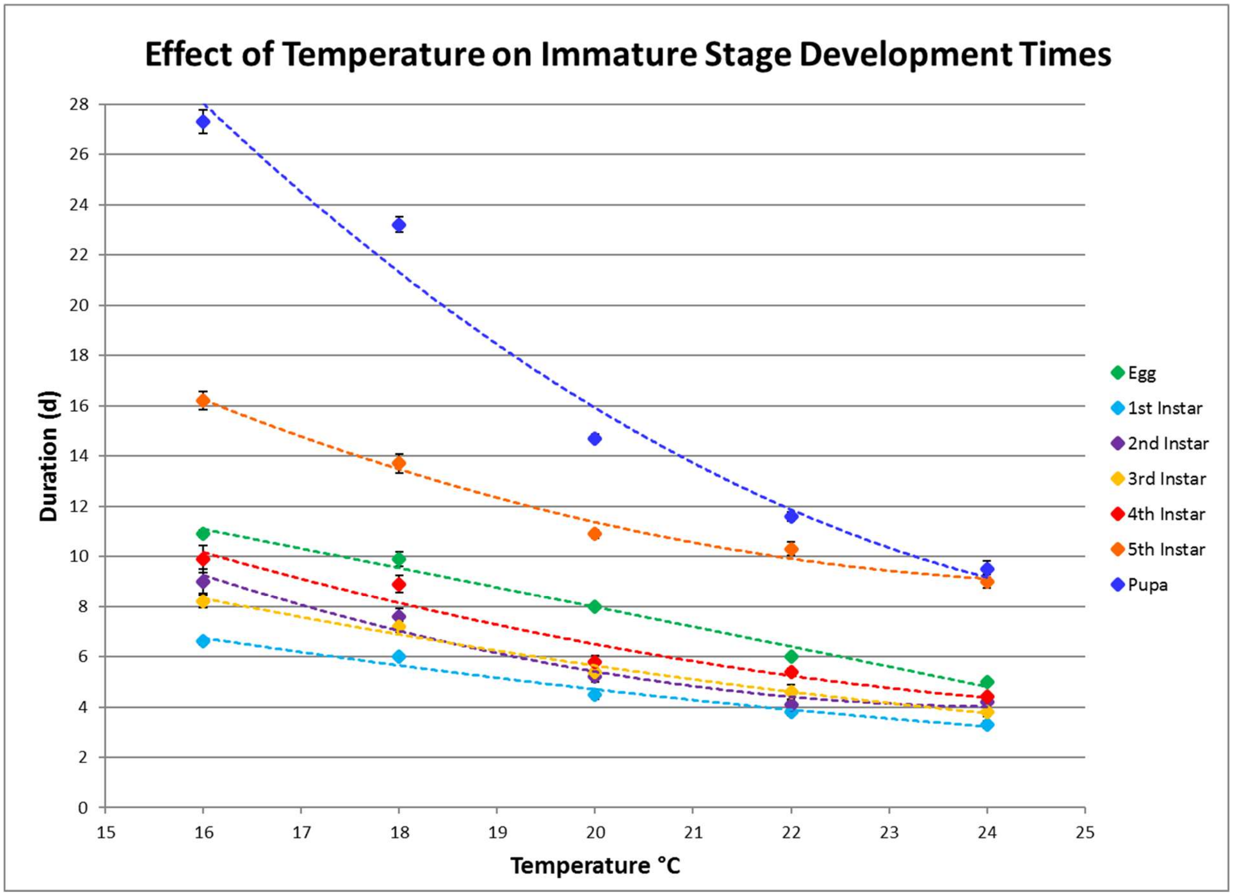

Figure 2.3. Quadratic trendlines and error bars (±) indicating the standard error of each point.

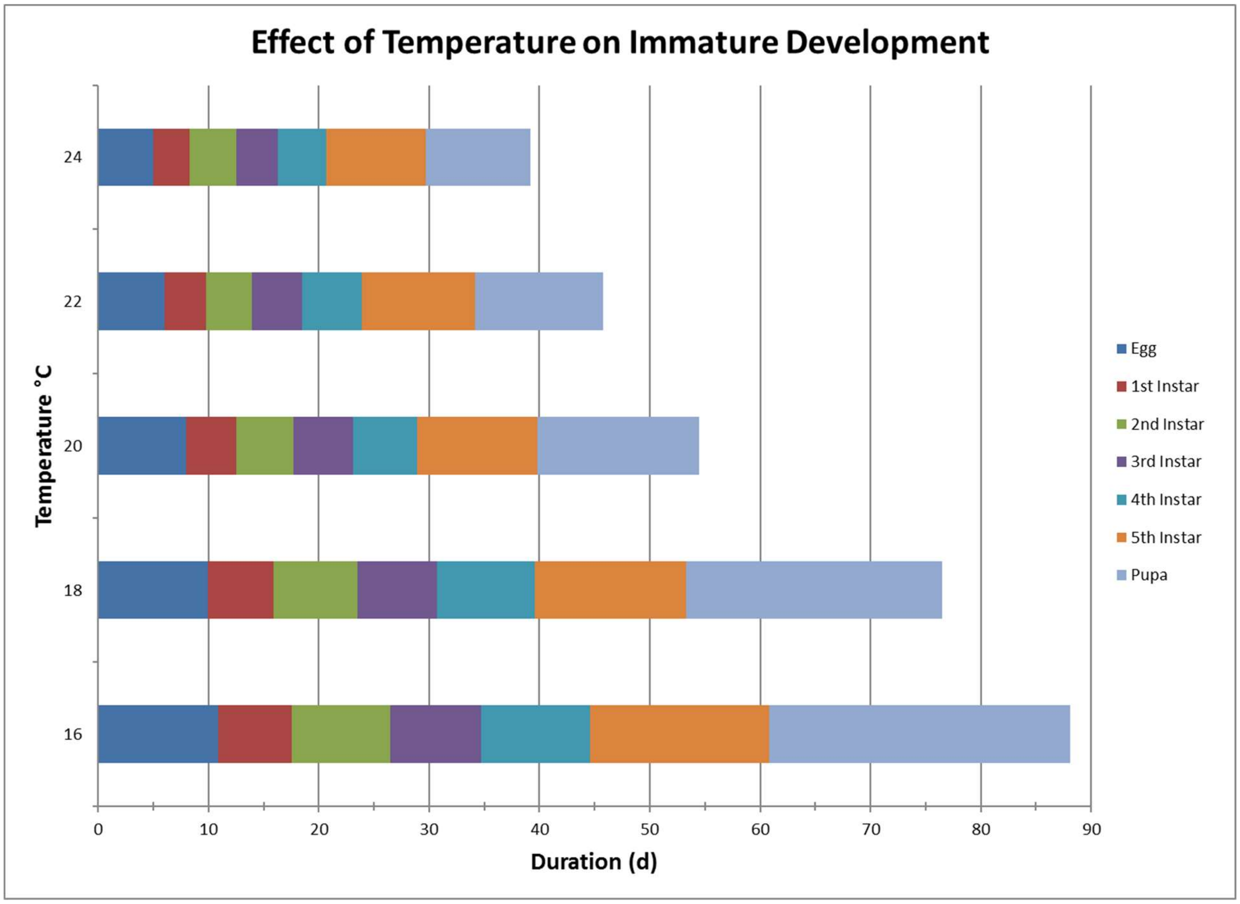

Figure 2.4. Comparison of immature stage growth at different temperatures. Development times of all immature stages decreased with increasing temperature. Total duration of immature stages was more than twice as long at 16°C than at 24°C.

Discussion

Given the proper conditions, the Kamehameha butterfly can be successfully reared in the laboratory for many generations using the above methodology. Once colonies are established, caterpillars can be raised, as long as sufficient food can be provided, and adults will mate given the right conditions, including natural lighting. Because only a relatively small number of adults in a cage typically mate, maintaining a large number of adults increases the chances that mating will occur to sustain a colony through multiple generations. Like with *V. atalanta* (Dimock, 1984), mating hours of *V. tameamea* can be extended by using lights right after sundown.

One of the most challenging aspects to maintaining colonies was keeping up with feeding the caterpillars and obtaining enough food to satisfy their large appetites, especially fifth instars. At times, over 1,000 caterpillars of various instars were maintained at the same time, which would consume two to three gallon-sized bags of māmaki leaves daily. Mass rearing would greatly benefit from the development of an acceptable artificial diet, though none of the diets tried were successful (W. Haines personal communication). A few commercially available artificial butterfly diets were modified and attempted by adding dried māmaki leaves to dry components (Morton, 1979), including those designed for *V. cardui* (Bioserv Inc.). Variations of Morton's (1979) diet recipes were also attempted, involving raw ingredients with added dried or fresh host plant (māmaki) foliage at different concentrations, but neither was accepted by the caterpillars.

As documented in *V. atalanta* (Dimock, 1984), rearing of caterpillars requires high humidity to maintain food freshness, but pathogens also thrive at high humidity, and can have devastating results. Using the above rearing methods, caterpillar mortality was most common when living conditions were unsuitable (poor quality or moldy foliage). Frequently, dead caterpillars would be found somewhat liquefied, or with a white fungus engulfing their bodies. Pathogen samples were not taken, so the identities of the disease(s) are not known. However, past observations of disease-infected caterpillars describe similar symptoms (Williams, 1928), and these are likely caused by a virus or bacterium. To avoid pathogen outbreaks, caterpillars were raised individually, and rearing cups were thoroughly sterilized with a bleach solution before being reused. Occasionally, caterpillars would be placed gregariously in plastic containers if food and usable cups were in short supply. In such cases, the majority of the caterpillars in the container would perish due to disease, especially those in the third, fourth, and fifth instar. Results from a separate study showed that mortality at 27°C was higher than those raised at 24°C (W. Haines unpublished data). It is possible that although higher temperatures can cause immature stages to develop quicker (Table 2.1), pathogens also reproduce more rapidly, especially in laboratory conditions where it is difficult to find a balance between maintaining air circulation and adequate humidity. For the main colony, immature stages were raised between 22-23°C, a temperature which appeared to balance the tradeoff between optimal growth and risk of pathogen outbreaks.

Captive Rearing and Release: Responsible Practices

It is hoped that the methodologies described in this chapter prove useful in future rearing projects involving this species and other Lepidoptera, particularly captive rearing and reintroduction efforts for the Kamehameha butterfly. However, these methods should be modified to avoid the negative effects captive reared individuals can have on wild populations. In conservation efforts for related species such as the monarch butterfly (Altizer et al., 2015), there have been concerns that captive rearing can lead to high levels of pathogens or more virulent strains being spread to wild populations (Cherry et al., 2005), introductions of pathogens to areas where they would otherwise not naturally occur (Meeus et al., 2011), inbreeding depression (Charlesworth & Charlesworth, 1987), loss in genetic diversity, and adaptations to captive conditions (Frankham, 2008). Since insects have short generation times and high fecundity, inbreeding depression and genetic adaptations can occur within a short period of time. These can include unintended adaptations to day length, light intensity, food sources, unnatural population densities, temperature, and absence of predators or other limiting factors (Altizer, 2015), which can lower the fitness of captive bred individuals when they are returned to the wild. For example, studies of monarch butterflies found that in comparison to wild adults, reared adults were significantly smaller, laid fewer eggs and had shorter lifespans (Flockhart et al., 2012; Lindsey et al., 2009). Captive bred individuals were also less likely to be recovered in their Mexican overwintering grounds (Steffy, 2015). In species with complex courtship behaviors, selection may favor less choosy individuals, causing the breakdown of these behaviors that can be important barriers to hybridization (Joron & Brakefield, 2003). Since mating success did appear to be a limiting factor in the colonies, with a relatively small percentage of adults mating in each generation, there is a strong chance that unintended selection behaviors were exerted or other traits that affected mating success. Scientific studies, like population monitoring, can also be negatively affected if surveys are being done close to the area of release, or if releases are made in an area where the animal does not naturally occur (Altizer, 2015).

Upon release of captive reared butterflies, the locations of source populations and introduction sites need to be taken into consideration to avoid genetic swamping or genetic pollution. When planning a reintroduction to a site, the source population should be as close as possible to the release site, certainly on the same island, and ideally in the same mountain range. When mass releasing butterflies into an area already containing a natural population, genotypes present in the native population can become diluted and may even be lost through genetic drift if the gene frequency is low (Fiedler & Kareiva, 2012). To mitigate this risk, Kamehameha butterflies should only be released into areas where they do not already occur (e.g. reintroductions into restored habitat), and numbers of released individuals should be high enough to facilitate establishment, but not so high that a large amount of spillover will occur into natural populations. If possible, there should also be buffer zones between reintroduction areas and areas containing native populations (Fiedler & Kareiva, 2012).

For captive rearing and release of the Kamehameha butterfly, sanitation protocols, such as washing rearing cups with bleach solution prior to reuse, and keeping tools and rearing areas disinfected, should be strictly implemented to prevent the establishment of new pathogen strains and the introduction of these pathogens to wild populations. Additionally, since caterpillars are not naturally gregarious, individuals should be kept separate as crowding will increase pathogen transmission and in turn lead to disease outbreaks in the laboratory. For releases, colonies should be kept in captivity for only the minimum number of generations necessary to raise sufficient individuals for release. Since this species can generate a large number of offspring in short amount of time, a surplus of adults can be produced in only two generations. For instance, even if a colony is started with only two wild females that lay fertile eggs, 300 or more offspring (150 per female) can feasibly be reared in the first generation. Although not all of these will survive to adulthood, and many will not successfully mate, if even 10-20 mating pairs can be produced from the first generation, one could realistically obtain 1000 or more larvae in the second generation, more than enough to attempt reintroduction to a site. Therefore, a colony should never be kept for longer than two generations, if the intention is to conduct releases into the wild. Along with minimizing the number of generations in captivity, Frankham (2008) comments that maintaining multiple fragmented populations can be an effective practice to avoid unwanted genetic adaptations and inbreeding depression. In addition, selection can be minimized by providing large enough spaces and making captive environments similar to those in the wild (Frankham, 2008). If care is taken to conduct captive rearing responsibly, and in consultation and cooperation with the Hawaii Department of Land and Natural Resources, these methods may be used to attempt to expand the range of this iconic butterfly beyond the upland forests where it is currently restricted. This may include releases into natural forests where host plants have been restored, or even into suitable residential areas with māmaki plantings in conjunction with protection from predators such as ants.

CHAPTER 3

EVALUATION OF PREDATION AND PARASITISM OF THE KAMEHAMEHA BUTTERFLY ON OAHU USING CONTROLLED-EXPOSURE TRIALS

Introduction

The Kamehameha Butterfly (Nymphalidae: *Vanessa tameamea*) is one of only two native butterflies to the state of Hawaii, the other being the Hawaiian blue (Lycaenidae: *Udara blackburni*). *V. tameamea* became Hawaii's official state insect in 2009, but sadly many local residents have never seen or even heard of the butterfly. Hawaii is extremely prone to invasion by non-native flora and fauna, which has led to the decline and even extinction of many of Hawaii's native species, and the state insect is no exception. Collection data and published records show that the Kamehameha butterfly was once common in wet and mesic areas throughout the islands, including the lowlands and relatively accessible areas such as Tantalus (Williams, 1928; Tabashnik et al., 1992). However, in recent years the butterfly is not nearly as widespread, and has been extirpated from many areas where it was once abundant (Tabashnik et al., 1992; Haines, 2014). A recent study, the Pulelehua Project, used field surveys and data submitted by "citizen scientists" to map distributions of the Kamehameha butterfly, and showed that its range has shrunk and shifted towards higher elevations in recent decades (Haines, 2014). It is unclear whether this range reduction is due to host plant declines, predation and parasitism by non-native species, or a combination of factors.

Impacts of Introduced Parasites and Parasitoids

A two-year conservation assessment conducted in the early 1980s on Hawaiian arthropods suggested that a number of factors might be responsible for insect declines, including the naturalization of non-native polyphagous parasites and predators (Gagne, 1982). Native species have evolved over millennia in the absence of many taxonomic groups, predisposing them to the impacts of introduced predators and competitors (Howarth, 1985). The impacts caused by alien parasitoids, including some of the 283 species introduced for biocontrol purposes between 1890 and 1985 (Funasaki et al., 1988), have been implicated as the primary cause for numerous extinctions and declines of native Lepidoptera (Gagne, 1982; Gagne & Howarth, 1985). While another study has questioned whether the non-target impacts of biocontrol releases are as severe (Funasaki et al., 1988), the fact that non-native parasitoids are impacting native Lepidoptera to some degree is certain (Henneman & Memmott, 2001; Asquith & Miramontes, 2001; Kaufman & Wright, 2010; King et al., 2010).

A few broad survey studies relating to parasitism of native moth species have been conducted in Hawaii (Gagne, 1982; Henneman & Memmott, 2001; Asquith & Miramontes, 2001). Surveys of parasitoids reared from wild moth larvae have indicated that non-native species, including those brought in for biocontrol, are having non-target effects on native Lepidoptera. For example, Henneman and Memmott (2001) collected over 2000 wild moth larvae (primarily native species) in Alakai Swamp, Kauai and found that of the parasitoids reared, 83% were non-native species purposefully introduced to control lowland Lepidoptera pests, 14% were accidentally introduced, and only 3% were native. A two-year survey using malaise traps conducted in Kokee State Park, Kauai also raised concern after finding that the majority of parasitoid wasps collected in their traps were introduced species (Asquith & Miramontes, 2001). Along with direct impacts to native Lepidoptera, the authors speculated that native parasitoids may also be indirectly impacted by competition for larval hosts, and suggested that all biological introductions of predators and parasitoids be halted.

Along with parasitoids, non-native predators such as birds and ants are major threats to native Lepidoptera. A large number of insectivorous alien birds have established in Hawaii, such as the Japanese white-eye (*Zosterops japonicas* (Temminck and Schlegel)) and common mynah (*Acridotheres tristis* (L.)). Gagne (1982) suggested that the demise of the native armyworm, *Agrotis crinigera* (Butler) (Noctuidae), was associated with the introduction of the common mynah. Ants worldwide are often major predators of Lepidoptera immature stages (Lach et al., 2016; Tvardikova & Novotny, 2012; Remmel et al., 2011) and have the potential to "exterminate entire populations" (Zimmerman, 1958). Hawaii is not thought to have any native ants, but nearly 60 ant species have established throughout the state ranging from urban to native areas, although many species are restricted to elevations below 900 m (Krushelnycky, 2015; Krushelnycky et al., 2005). Because the native flora and fauna are believed to have evolved without ants, native insects are thought to be extremely susceptible to the direct effects of ant predation, as well as indirect effects caused by ants, including alterations to their habitat and food sources (Krushelnycky et al., 2005). Ants are believed to be responsible for the decline of numerous Hawaiian arthropods (Medeiros et al., 1986; Howarth, 1985; Loope et al., 1988; Daly & Magnacca, 2003), including the extinction of the noctuid *Agrotis fasciata* (Rothschild) (Lepidoptera: Noctuidae) from Midway Atoll (Gagne, 1982).

Other studies quantifying predation rates of Lepidoptera larvae have found that ants and birds can have high impacts. Lach et al. (2016) found that after deploying second instar larvae of *Vindula arsinoe* (Nymphalidae), a butterfly native to Australia and in the same family as the Kamehameha butterfly, *A. gracilipes* attacked at much higher rates than the native tree ant, *Oecophylla smaragdina* (Formicidae). In another study authors deployed approximately 14,000 artificial larvae either exposed on leaf surfaces or concealed in leaf folds and found that ants and birds had the highest attack rates overall, and exposed sentinel larvae were significantly more susceptible to predation (Tvardikova & Novotny, 2012). Similar studies comparing free-living and semi-concealed caterpillars found ants to impact both types (Fowler & Macgarvin, 1985) equally, whereas Ito and Higashi (1991) found *Formica* species to impact free-living Lepidoptera larvae more than leaf-folding larvae on birch trees. Although the impacts caused by birds and ants are difficult to quantify, the fact that they pose a threat to many Lepidoptera species worldwide is undeniable.

Previous Controlled-exposure Trials

Although field sampling and rearing wild larvae can provide a good assessment of which parasitoids are most common, it does not provide specific information regarding their role and impacts in the ecosystem (Kaufman & Wright, 2009). By using sentinel larvae, an artificial population can be introduced for a designated period of time, and the mortality factors relating to it can be monitored in a more controlled setting. Sentinel larvae have been deployed in the field through a number of methods. In controlled-exposure treatments (Kaufman & Wright, 2009; King et al., 2010; Holmes et al., 1979; Lach et al., 2016) larvae are deployed in different treatments that either allow or exclude predators or parasitoids. By comparing rates of survival, parasitism, or disappearance, the effect of predators and parasitoids on the natural population can be quantified. Artificial larvae have also been used in controlled-exposure trials. Characteristic markings imbedded on the artificial larvae by attempts at predation and parasitism were analyzed to identify the types of predators and parasitoids attacking wild larvae (Tvardikova & Novotny, 2012). Another study deployed individual larvae for the duration of their current instar to estimate parasitism and disease rates of each instar (Boettner et al., 2000). The same study deployed groups of first instar larvae which were monitored daily until pupation for mortality factors and disappearance over the course of the entire larval stage.

The majority of studies on conservation of Hawaiian Lepidoptera have focused on native moths, a number of which have been labeled as endangered, extinct, or in decline (Gagne, 1988; Gagne & Howarth, 1985; Rubinoff & San Jose, 2010). Despite much speculation on the impacts of non-native parasitoids and predators, there have been only two controlled-exposure studies conducted in the Hawaiian Islands on native Lepidoptera (Kaufman & Wright, 2009; King et al., 2010), both focusing on moth species in the family Crambidae. This study will be the first on a butterfly in Hawaii.

Previous Information on Predation and Parasitism of the Kamehameha butterfly

Most previously known information on threats to the Kamehameha butterfly is based on collection and rearing of caterpillars (Leeper, 1975; Tabashnik et al., 1992; Williams, 1928; Gorelick & Wielgus, 1968). Information documenting larval, egg, and pupae parasitoids from the Proceedings of the Hawaiian Entomological Society were compiled by Leeper (2014) and are summarized in Table 3.1. Additional mortality factors, other than the observation of a pathogen causing mortality to the larval stage (Williams, 1928), have not been formally documented. Based on specimens in museums across the state and information regarding its past and present distribution, the butterfly is believed to be declining statewide (W. Haines unpublished data), and is now absent in areas where it was once abundant such as the Tantalus region of Oahu (Tabashnik et al., 1992) and other parts of the Southern Koolau Range. To begin understanding the reason for its recent decline, controlled-exposure trials were conducted on Oahu using sentinel eggs and larvae.

Table 3.1. A list of known parasitoids compiled by Leeper (2014). Two parasitoids (*Chaetagaedia monticola* & *Brachymeria obscurata*) infect the larva stage but do not emerge until the chrysalis stage. *Pteromalus puparum* may go through the same type of lifecycle, but it has not yet been clearly stated for this species.

Order	**Family**	**Genus & Species**	**Stage Affected**	**References**
Diptera	Tachinidae	*Chaetogaedia monticola* (Bigot)	Larva, em. from chrysalis	Swezey, 1927
Hymenoptera	Chalcididae	*Brachymeria obscurata* (Walker) (=*Chalcis obscurata* Walk.)	Larva, em. from chrysalis	Funasaki et al., 1988 Swezey, 1922 Williams, 1928
Hymenoptera	Ichneumonidae	*Echthromorpha fuscator* (Fab) (=*Echthromorpha maculipennis* Holmgren)	Chrysalis	Swezey, 1912, 1915, 1929
Hymenoptera	Pteromalidae	*Pteromalus puparum* (Linnaeus)	Chrysalis	Swezey, 1931
Hymenoptera	Trichogrammatidae	*Trichogramma minutum* Riley	Egg	Swezey, 1929, 1934
Hymenoptera	Trichogrammatidae	*Trichogramma perkinsi* Girault (=*Pentarthron flavum* Perk.)	Egg	Swezey, 1913, 1915

Study Objectives

Hawaii supports remarkably high endemism in both flora and fauna, and because the Kamehameha butterfly is one of only two native butterflies in the archipelago, it represents a unique component of biodiversity. The reasons for its decline might be due to a number of factors. Bottom-up factors, such as host plant rarity, might be significant, but are not thought to be the only factor in the decline of the butterfly because its primary host plant, māmaki, is still relatively common in some areas that the butterfly previously populated (Tabashnik et al., 1992). Top-down factors, such as predation and parasitism, have been found to affect Lepidoptera populations in Hawaii and in other regions (Krushelnycky et al., 2005; Kaufman & Wright, 2010; King et al., 2010; Henneman & Memmott, 2001). This study aims to provide insight on Kamehameha butterfly decline by quantifying impacts of top-down factors, specifically parasitoids, ants, and birds, on immature stages of the butterfly. Field studies were conducted on Oahu at sites where the butterfly is extant and sites where the butterfly has been extirpated, despite the host plant being present. Results from this study will shed light on what factors are affecting populations of the Kamehameha butterfly and what needs to be done to increase the likelihood of success of recolonization or reintroduction. It will also provide a basis for similar studies on predation and parasitism of other Lepidoptera.

Methods

Study Sites

Four sites on Oahu Island were selected for the study. Sites were selected based on the availability of suitable host plants, feasibility of access, and status of the butterfly (extant or extirpated). Kahanahaiki Valley (537-580 m elevation) and Palikea Trail (849-921 m), both in the Waianae Range, both supported a stable butterfly population. Manoa Cliff Trail (558 m) and Lyon Arboretum (149-213 m), the two sites in the Koolau Range, have formerly supported Kamehameha butterfly populations, but have not since the early 1990s based on museum specimens and observations by naturalists. Differences in predation and parasitism were anticipated between the sites based on the status of the butterfly and other factors such as elevation and degree of invasion by non-native fauna and flora.

Prior to deployments, sites were surveyed for ants and other egg predators such as katydids. The presence and identity of ants was determined using baited vials containing peanut butter, canned tuna, and honey. Two vials were placed at the base of each māmaki tree used in the field trials, and left undisturbed for 45-60 minutes before being collected. Temperature data were also recorded at each site using a data logger (HOBO Pro v2 2x external temperature data logger, Onset Computer Corporation), to estimate the development time of the egg and larval instar stages at each site. Estimating realistic development times at each site was necessary to accurately estimate risk of predation and parasitism over the duration of larval development. Temperature probes were affixed to māmaki branches at heights of 1 m and 2 m and left in place for at least 72 hours. Temperature probes were deployed during March (Manoa Cliff Trail and Lyon Arboreutm), April (Palikea Trail), and May (Kahanahaiki Valley) of 2016.

Laboratory Colonies

All caterpillars and eggs used for deployment trials were reared in captivity. Because deployment sites were in both the Koolau and Waianae mountain ranges, a colony for each mountain range was established in the laboratory and kept separate to prevent genetic mixing. Both colonies were started using wild caught females. The Koolau colony originated from butterflies collected in Kahana Valley, and the Waianae colony originated from butterflies collected in Waianae Valley near the start of the Waianae-Kaala Trail. Occasionally, additional genetic stock was introduced to the Waianae colony from Palikea and Kahanahaiki Valley, but not to the Koolau colony because butterfly populations in that mountain range were not as easily accessible, and attempts to obtain additional stock were not successful. Wild caught females were brought back to the laboratory where they were placed in individual mesh cages measuring (60.5 cm x 35 cm x 35 cm), and supplied mashed fruit, paper towels saturated in sugar and electrolyte solution, and a potted māmaki plant for oviposition. Eggs were collected daily and stored in plastic cups until hatching (typically 6 days at 22°C). Hatchlings were separated into individual cups with māmaki foliage until used for deployment. See Chapter 2 for detailed rearing procedures.

Egg Deployment

To assess egg parasitism and predation, 844 sentinel eggs were deployed. Only eggs laid within 24 h were deployed to prevent hatching in the field (typical duration of the egg stage is 6 days), and because trichogrammatid egg parasitoids tend to prefer ovipositing in eggs early in their development (Monje et al., 1999; Ruberson & Kring, 1993). On some occasions, when daily egg production was low, freshly laid eggs were stored in a growth chamber at 10°C chamber for less than 48 hours so that enough eggs could be accumulated for deployment. Chilling eggs for short periods temporarily slowed development but did not appear to negatively affect viability. Each egg was glued with non-toxic glue (Elmer's Glue-All) to strips of wax paper under a dissecting microscope, with the micropyle facing up. This glue was selected due to its lack of strong scent and use in other studies involving egg parasitism (Atanassov et al., 2003;

McGregor et al., 2000). Once the glue set, the wax paper was cut into squares of approximately 1mm^2, each containing a single egg.

At field sites, the wax paper squares containing the eggs were glued to leaves of wild māmaki at each of the four sites. Eggs were deployed in pairs, with one egg glued to the upper surface of a leaf and the other to the lower surface of the same or a nearby leaf. Eggs were glued on both sides of the leaves since wild eggs were observed on both the surface and underside. Depending on the size of the māmaki tree and availability of usable leaves, one to four pairs were deployed per tree. Eggs were left in place for 72 h. Each egg was assigned a number and its height and location on the leaf (upper or lower surface) were recorded. Upon retrieval, the fate of the egg (presence or absence of the egg and wax paper) was recorded. In cases when remnants of the shell remained, and there was clear evidence that the egg had been chewed, I considered the egg to be predated. In cases where the egg was completely missing from the wax paper with no shell remaining, or where the wax paper itself was missing, I considered this to be an ambiguous result, and excluded these eggs from the analyses. This generally occurred after periods of heavy rain, and it is likely that eggs or wax paper had been washed from the leaves. All retrieved eggs were brought back to the laboratory to be reared for caterpillars and/or parasitoids.

During egg deployments, ants and other predators were generally not excluded because the primary goal was to assess egg parasitism. However, during initial trials at Lyon Arboretum, more than half of deployed eggs were not recovered after three days, and most of these showed obvious chewing marks indicating predation. At this site, along with ants (the white-footed ant *Technomyrmex* sp. nr. *albipes* and the yellow crazy ant *Anoplolepis gracillipes*), a non-native katydid *Conocephalus saltator* Saussure (Tettigoniidae) was abundant at times. This katydid is primarily carnivorous, and other members of the genus are important predators of small insects and Lepidoptera eggs even to the extent of providing biological control of pest caterpillars (de Kraker, 2000; Illingworth, 1931). Another orthopteran, the cricket *Nanixipha nahoa* Carvalho & Shaw (Trigonidiidae), was also abundant on māmaki at the site. Therefore, a small trial was done to assess whether egg predation was caused by ants or larger predators such as the katydid. Forty eggs were deployed at heights ranging from 1 m to 2 m, in a paired experiment in which half were deployed with netting over them that excluded katydids and crickets, but allowed entry of ants. Egg survival was assessed after three days.

Controlled-exposure Treatments

Larval deployments occurred at all four sites and included all five instars. Only freshly molted caterpillars were deployed to ensure they remained the same instar during the entire three-day duration of deployment. Two to three days prior to deployment, colony caterpillars were segregated by instar and monitored for molting. The day before deployment, newly hatched (first instar) or newly molted (instars 2-5) caterpillars were separated to be used the following day for deployment.

To quantify the impacts of parasitoids and predators, 1509 larvae were deployed on wild māmaki branches under three different treatments over the course of two years. One treatment excluded both birds and ants, but allowed parasitoid entry. This treatment consisted of an exclosure (30 cm diameter x 50 cm length) fashioned of bird netting (Easy Gardener BirdBlock, 15.9 mm mesh) covering the branch on which the caterpillar was placed, as well as a sticky resin (Tree Tanglefoot) spread over applied to the base of the branch. The second treatment was similar but only included the bird netting, excluding birds but allowing entry of ants and parasitoids. The third treatment was an open treatment and was simply a branch with no exclosure or resin barrier, so caterpillars were exposed to all predators and parasitoids. Caterpillars were deployed in sets of three, with one caterpillar in each of the three conditions on the same tree. All caterpillars in a set were of the same instar. Each of the three treatments were haphazardly assigned to branches, and were separated by at least 1 m. To increase the likelihood that caterpillars would remain on their assigned branches, only branches that appeared healthy and full of leaves were selected for treatments. Depending on the number of suitable branches, one to three sets of caterpillars were deployed on each tree. Each branch containing a caterpillar was tagged with a label on horticulture tape. The instar, height and treatment were recorded for each caterpillar upon deployment. Caterpillars were left in place for 72 hours and their presence, absence, and current instar was recorded upon retrieval. Any missing caterpillars were diligently searched for on nearby branches in case they had migrated, although most caterpillars that were recovered remained on the same branch where they were deployed. All retrieved caterpillars were brought back and reared in the laboratory until adulthood to assess parasitism. Any dead caterpillars were inspected daily for five days after death in case of delayed parasitoid emergence.

Data Analysis

To estimate the impacts of each mortality factor (birds, ants, and unknown disappearance), larval disappearance rates between treatments for each site and instar were compared. The effect of birds and ants over the three-day deployment period was determined using the difference in larval disappearance between treatments. The effect of ants was estimated by subtracting the disappearance rate in the “bird and ant excluded” treatment from the disappearance rate in the “bird excluded” treatment. Likewise, to estimate the effect of birds, the disappearance rate in the “bird excluded” treatment was subtracted from the disappearance rate in the “open” treatment. Larval disappearance data was also interpreted using calculated risk-values and compared between sites and treatments using binomial logistic regression. Since larvae and eggs were only deployed for three-day periods and sites varied in elevation and temperature, equations were used to estimate the risk of larval disappearance and egg parasitism based on each stage’s period of susceptibility at each site (King et al., 2010). Using the temperature data collected from field sites (Table 3.2) and the relationship between temperature and development times established in Chapter 2 (Table 2.3), durations of the egg stage and each instar were estimated for each site (Table 3.2). Larvae were considered to be susceptible to disappearance for the total estimated duration of each instar stage at the respective site. The susceptibility of eggs to parasitism was considered to be half that of the total duration of the egg stage at the respective site based on previous laboratory trials that found *V. tameamea* eggs to be vulnerable to parasitism by *Trichogramma* only for the first half of their development (W. Haines & E. Magarifuji, unpublished data).

Comparisons of larval parasitism among sites or instars were not conducted since almost no larval parasitism was detected. Eggs were deployed at all four sites, but egg parasitism was only detected at Palikea Trail. Egg parasitism data at Palikea were analyzed by calculating risk-values and compared among sites and placement (top or bottom of leaf) using binomial logistic regression.

Risk of Predation and Parasitism

Risk-values or *r-values,* have been used to estimate the probability of a specific event occurring due to certain factors (King et al., 2010). Here, the risk of disappearance of deployed larvae due to bird and ant predation, and the risk parasitism of deployed eggs by a parasitic wasp, is being estimated. Methods similar to those employed by King et al. (2010) were used to calculate risk-values, the risk of predation or parasitism, for specific life stages as well as cumulatively over the course of larval development. Initially, a risk-value was calculated for each instar of each treatment at each site using Eq.1. Using similar methods, egg parasitism was analyzed using Eq.1. Palikea Trail was the only site where egg parasitism was observed, and only eggs that were successfully retrieved were used to estimate risk of parasitism. Risk-values were also used to estimate the effect of ants (Eq.2a) and birds (Eq.2b), and to estimate survival to pupation using simulated cohorts if larvae were protected from birds and/or ants. Furthermore, the cumulative effect of birds and ants (Eq.4a, b), and cumulative risk of larval disappearance (Eq.3) were calculated. When comparing larval disappearance rates using risk-values, Kahanahaiki Valley was divided into two areas separated by about 150 meters; one was infested with the yellow crazy ant (*Anoplolepis gracilipes*) while the other was not. This allowed examination of the effects of the yellow crazy ant specifically.

In the equations below, n corresponds to the life stage (e.g. instar) of deployed eggs or caterpillars being analyzed. e_n was the number of days that group n was deployed, p_n was the actual proportion of disappearance (larvae) or parasitism (eggs) found during the deployment period, and d_n was the calculated duration, in days, that life stage n would be susceptible to predation or parasitism. For larval instars, the duration of susceptibility (d_1, d_2, d_3, d_4, and d_5) was simply the estimated duration of that life stage at each site. For eggs, the duration of susceptibility (d_{egg}) was considered to be half of the estimated duration of the egg stage at the site.

Eq.1. $r_n = 1 - (1 - p_n)^{d_n/e_n}$

Eq.2.

a. Ant effect for life stage n: $A_n = r_{n\,\text{(bird excluded)}} - r_{n\,\text{(bird and ant excluded)}}$

b. Bird effect for life stage n: $B_n = r_{n\,\text{(open)}} - r_{n\,\text{(bird excluded)}}$

Eq.3.

Cumulative risk of larval disappearance:

$$r_{\text{cumulative}} = 1 - [(1 - p_1)^{(d_1/e_1)} * (1 - p_2)^{(d_2/e_2)}(1 - p_3)^{(d_3/e_3)} * (1 - p_4)^{(d_4/e_4)} * (1 - p_5)^{(d_5/e_5)}]$$

Eq.4.

a. Cumulative ant effect:

$$A_{\text{cumulative}} \quad r_{\text{cumulative (bird excluded)}} - r_{\text{cumulative (bird and ant excluded)}}$$

b. Cumulative bird effect:

$$B_{\text{cumulative}} \quad r_{\text{cumulative (open)}} - r_{\text{cumulative (bird excluded)}}$$

To illustrate the impact of ants and birds on caterpillars over the course of an entire life cycle, I graphed simulated survivorship curves for cohorts of 200 caterpillars (the approximate number of eggs that can be laid by single Kamehameha butterfly female over her life span). Using the risk-values (r_n) to estimate the mortality for each instar, survivorship curves were simulated for each site and treatment.

Binomial Logistic Regression

Binomial logistic regression is used to test for differences in the probability of a dichotomous dependent variable (often the likelihood of an event) based on one or more independent variables (Sokal & Rohlf, 2011). In this study, the dichotomous dependent variable was the retrieval (Yes) or disappearance (No) of a caterpillar, or presence (Yes) or absence (No) of egg parasitism, following a 3-day period of deployment. For larvae, the independent variables of interest were treatment, site, instar, and (for Kahanahaiki only) presence or absence of yellow crazy ants. For eggs, the independent variables of interest were site and location of egg (top or bottom of leaf). Pairwise comparisons were conducted by running repeated binomial logistic regressions using each value of the independent variable (treatment, site, instar) as the reference value followed by a Bonferroni adjustment for multiple comparisons. Statistical tests were not conducted on larval parasitism since it was only detected at one site. Binomial logistic regression analyses were conducted in Minitab 14.

Identification of Parasitoids

Hymenopteran egg and larval parasitoids, which were recovered only at Palikea, were identified using genetic data generated by polymerase chain reaction (PCR) followed by Sanger sequencing. Using a Qiagen DNeasy Blood and Tissue Kit and standard protocols, DNA was extracted from adult trichogrammatid egg parasitoids as well as a hymenopteran larval parasitoid that died before pupation. Whole specimens were used for extractions. Both taxa were sequenced for the “barcoding” region of CO1 (660 bp) using the primers LCO1490 (5’ GCTCAACAAATCATAAAGATATTGG 3’) and HCO2198 (5’ TAAACTTCAGGGTGACCAAAAAATCA 3’) (Folmer et al., 1994) with an annealing temperature of 55°C. Trichogrammatid parasitoids were also sequenced for a 550 bp region of ITS-2 commonly used to identify Trichogrammatidae, using the primers ITS-2F (5’ TGTGAACTGCAGGACACATG 3’) and ITS-2-R-Trich (5’ GTCTTGCCTGCTCTGAG 3’) (Davies et al., 2006) with an annealing temperature of 53°C. Sequences were aligned, trimmed, and queried against the online databases Genbank (www.ncbi.nlm.nih.gov/genbank/) and the Barcode of Life Database (Ratnasingham & Hebert, 2007) to identify the closest matches based on sequence similarity.

Results

Site Characteristics

Table 3.2 shows the egg and larval developmental times per site. Developmental times were estimated using the quadratic trendlines from Table 2.3 and average temperatures measured at each site.

Table 3.2- Estimated egg and larval development times at each site.

				Instar (d)					
Site	Elevation (m)	Mean Temp. (°C)	Egg (d)	1st	2nd	3rd	4th	5th	Total (d)
Lyon Arboretum	149-213	22.1	6.3	3.10	4.4	4.6	5.2	9.8	27.8
Manoa Cliff Trail	558	18.6	9.1	5.4	6.5	6.5	7.6	12.8	38.7
Kahanahaiki Valley	537-580	18.5	9.1	5.4	6.6	6.6	7.7	12.9	39.1
Palikea Trail	849-921	16.7	10.5	6.4	8.4	7.8	9.4	15.2	47.1

No ant species were detected beneath study trees at Manoa Cliff Trail and Palikea Trail. All other sites were inhabited by at least one species of ant, and some sites were inhabited by *A. gracilipes* (yellow crazy ant), which is considered to be highly invasive in Hawaii (Krushelnycky et al., 2005; Krushelnycky, 2015; Plentovich et al., 2011; Lach et al., 2016; Holway et al., 2002; Table 3.3).

Table 3.3- Ant species detected at each site.

Site	Ant Species Detected
Lyon Arboretum	*Anoplolepis gracilipes*, *Technomyrmex difficilis*
Manoa Cliff Trail	None detected
Kahanahaiki Valley area w/YCA	*Anoplolepis gracilipes*, *Solenopsis papuana*
Kahanahaiki Valley area w/no YCA	*Solenopsis papuana*
Palikea Trail	None detected

Egg Deployments

Egg Predation

A total of 816 eggs were deployed across the four sites. Of these, 497 (61%) were retrieved intact, 60 (7%) were found with obvious chewing marks to the egg shell, and were thus considered predated, and 259 (32%) were entirely missing, and were presumed to have been washed off of leaves during periods of heavy rain (Table 3.4). For instance, during one deployment at Kahanahaiki Valley in May 2016, immediately after which there were heavy rains, 84% (57 of 68) of the eggs deployed disappeared with no wax paper or egg shells remaining, and these were assumed to have washed off. The highest percentage of intact-eggs were retrieved from Palikea Trail (77%). However, 35% (83 of 236) of these retrieved eggs were found to be parasitized (Table 3.4).

The highest percentage of eggs observed with obvious chewing marks was at Lyon Arboretum (26%, Table 3.4). A small trial at Lyon Arboretum testing possible egg predation by katydids found that 100% of the 20 eggs deployed with mesh covering them were retrieved intact, while 8 (40%) of the 20 eggs deployed simultaneously without mesh were clearly predated. This small study along with no-choice lab testing with the two abundant orthopteran species, *C. saltator* and *N. nahoa*, provided us with evidence that these species could potentially be major egg predators to the Kamehameha butterfly. Capturing footage of the katydid species feeding on the eggs was unsuccessful.

Table 3.4. Egg predation and parasitism across the four sites.

Site	Total Deployed	Total Retrieved (incl. parasitized)	Total Missing (incl. predated)	Total Parasitized	Total Predated
Lyon Arboretum	157	64 (41%)	93 (59%)	0	41 (26%)
Manoa Cliff Trail	144	96 (67%)	48 (33%)	0	9 (6%)
Kahanahaiki Valley	208	101 (49%)	107 (51%)	0	5 (2%)
Palikea Trail	307	236 (77%)	71 (23%)	83 (35%)	5 (2%)
Grand Total	**816**	**497 (61%)**	**319 (39%)**	**83**	**60 (7%)**

Egg Parasitism

Egg parasitism was only observed at Palikea Trail, and only a single species of egg parasitoid was reared: a trichogrammatid wasp. The species determination is pending, but DNA sequence data and morphological characteristics place it in the genus *Trichogramma*, which includes both native and non-native species in Hawaii. Mitochondrial (CO1) and nuclear (ITS2) sequences of the egg parasitoid were not a species-level match to any species represented in the online DNA databases (BOLD and Genbank); the closest match for CO1 was *T. platneri* (92%, accession number KX512841.1) and the closest match for ITS-2 was *T. pintoi* (85%, accession number JF920460.1). Because the online databases include ITS-2 and CO1 sequences from most of the non-native *Trichogramma* spp. that have been recorded from Hawaii (Nishida, 2002), it seems likely that the reared species is a native *Trichogramma*, none of which have been sequenced. The native *T. perkinsi* has been reared from *V. tameamea* eggs collected on Oahu (Swezey, 1913; 1915), and morphologically the specimens appear to be a good match for this species, but because *Trichogramma* are difficult to identify and have few diagnostic characters, the specimens must be examined by a specialist.

Binomial logistic regression found the likelihood of parasitism at Palikea Trail to be highly significantly higher than the other sites ($p<0.000$). Out of 307 eggs deployed over five different deployments (September 2015, November 2015 twice, March 2016, April 2016), 77% (N=236) eggs were retrieved and used to calculate parasitism rates. Parasitism rates varied over time (Fig. 3.1), with the highest parasitism occurring in late March 2016 (81%, N=34) and early April 2016 (56%, N=40). In November 2015, 9 (9%) of the 99 retrieved were parasitized, and in September 2015, none of the 23 retrieved were parasitized. A total of 83 (35%) eggs were parasitized (Table 3.4).

Risk-values were used to estimate the overall risk of egg parasitism over their entire period of susceptibility (estimated to be 5.3 days at Palikea). Using *Eq.1*, a risk-value of 0.533 was calculated for egg parasitism at Palikea Trail.

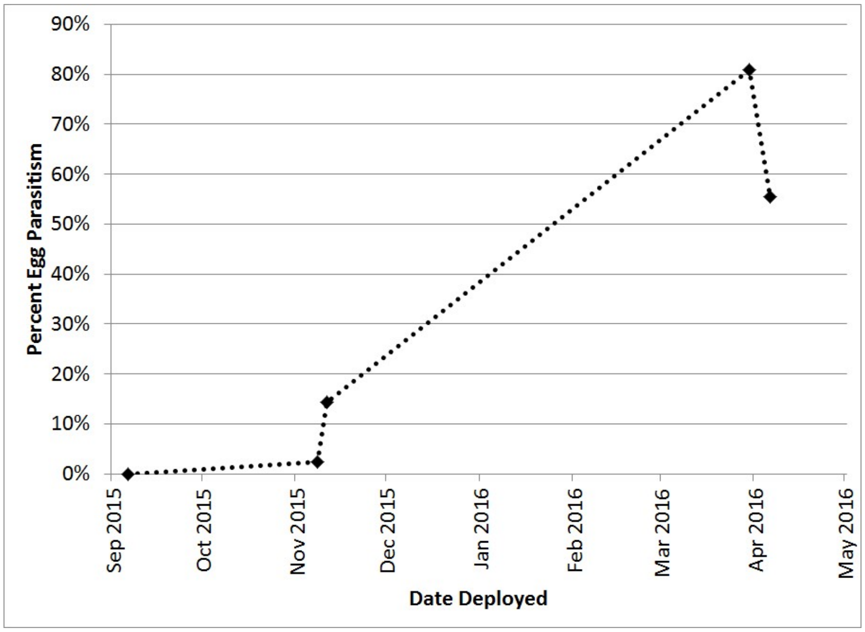

Figure 3.1 Percentage of retrieved eggs from Palikea Trail that were parasitized over the course of five deployments.

Larval Deployments

Larval Parasitism

Parasitoids were recovered from only two caterpillars during the course of this study; both were second instar caterpillars deployed in different areas of Palikea on the same date, from 11-14 Nov 2015. However, these parasitoids emerged from caterpillars as larvae and died without developing further. Both were Hymenopteran larvae and were similar in appearance. The larvae yielded good quality sequence data from CO1, and the closest identified match (93.1% similar) was a specimen of *Oncophanes* sp. (Braconidae: Rhyssalinae) collected in Ontario, Canada (BOLD sample ID BIOUG02604-B03). To my knowledge, there are no *Oncophanes* spp. established in Hawaii, nor are there any braconids in the subfamily Rhyssalinae (Nishida, 2002; Beardsley, 1961). However, there are over 70 species of braconid established in Hawaii, mostly purposefully or accidentally introduced nonnative species. There are three endemic braconid species (*Aspilota konae* Ashmead, *Ecphylopsis nigra* Ashmead, and *E. swezeyi* Beardsley), but none of these are represented in online sequence databases. Because the larval parasitoids died before reaching adulthood, they could not be identified using morphological characters.

Larval Disappearance

A total of 1509 larvae were deployed across the four sites (Lyon Arboretum: N =369, Manoa Cliff Trail: N =339, Kahanahaiki: N =453, Palikea: N =348) (Table 3.5). The percentage of larvae that disappeared varied greatly among instars, treatments, and sites. When all sites are pooled, trends can be seen among treatments and instars. For all instars, the open treatment consistently showed the highest percentage (38% to 58%) of disappearance, and the treatment excluding birds and ants showed the lowest (13% to 24%, Fig. 3.2). A significant difference ($p < 0.0167$) between the “open” and “bird and ant excluded” treatments was found for instars 1, 3, 4, and 5. Using a binomial logistic regression model that took into account variation due to site and treatment, I found that fourth and fifth instars disappeared significantly more often ($p < 0.005$) than first and third instars, and there was no significant difference between disappearances of first, second, and third instars (Fig. 3.2). Lastly, a highly significant difference ($p < 0.000$) was found between all three treatments when instars and sites are pooled.

Table 3.5. Breakdown of total larvae deployed at each site, for each instar, and each treatment.

Site	Instar	Open	Bird Exc.	Bird & Ant Exc.	Grand Total
Manoa Cliff Trail	1	40	18	38	96
	2	28	14	26	68
	3	25	13	26	64
	4	28	7	28	63
	5	20	11	17	48
	Total	135	63	141	339
Kahanahaiki Valley	1	38	24	32	94
	2	44	25	33	102
	3	38	23	32	93
	4	32	34	34	100
	5	21	22	21	64
	Total	173	128	152	453
Lyon Arboretum	1	39	9	30	78
	2	41	13	28	82
	3	37	15	29	81
	4	20	11	13	44
	5	45	10	29	84
	Total	182	58	129	369
Palikea Trail	1	17	17	17	51
	2	29	19	24	72
	3	30	21	22	73
	4	34	20	26	80
	5	26	20	26	72
	Total	136	97	115	348
Grand Total		632	346	531	1509

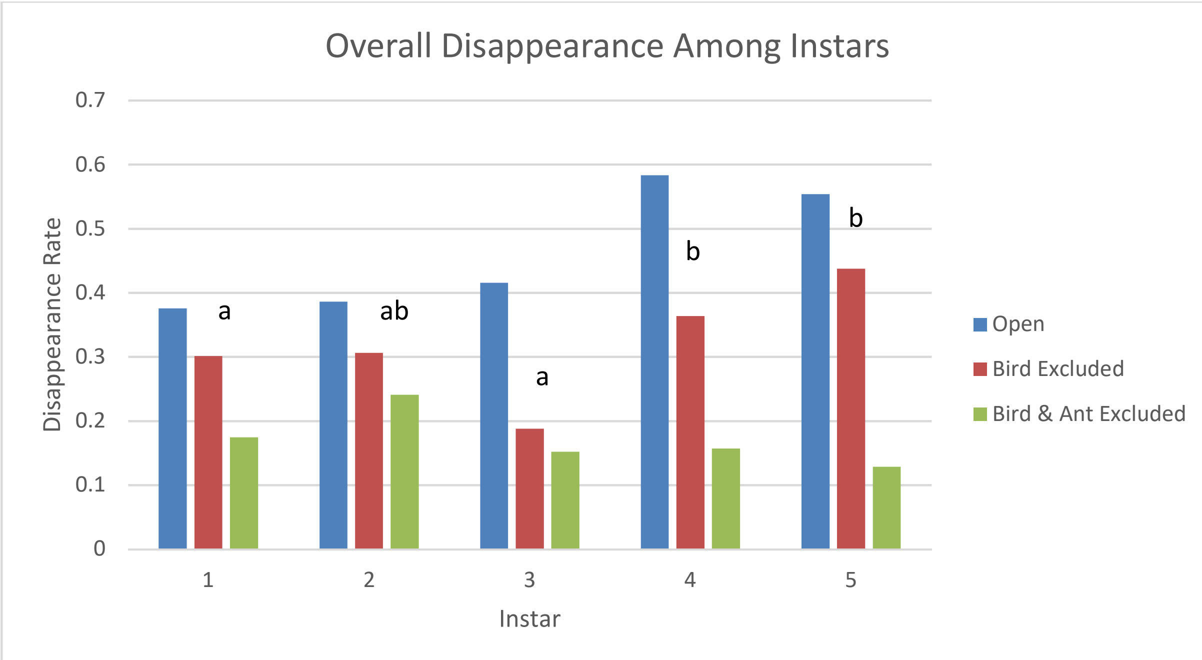

Figure 3.2. Disappearance rates among instars when all sites are pooled together. Identical letters indicate no significant difference in overall disappearance between instars based on binomial logistic regression. Different letters indicate that instars were significantly different after Bonferroni adjustment for multiple pairwise comparisons ($p<0.005$).

When instars are pooled, and sites and treatments are compared, the same trend between treatments can be seen (Fig. 3.3). For all sites, the disappearance in the "open" treatment was significantly higher ($p < 0.0167$) than the "bird and ant excluded" treatment. Furthermore, the "open" treatment was significantly higher ($p < 0.0167$) than the "bird excluded" treatment for Kahanahaiki Valley, Lyon Arboretum, and Palikea Trail. However, at Palikea Trail, where ants were not detected, the rates of disappearance in the "bird and ant excluded" treatment and the "bird excluded" treatment were essentially equal. When looking at overall disappearance after taking into account variation due to instar and treatment, Palikea Trail had a significantly lower ($p < 0.0083$) disappearance rate than other sites, and Kahanahaiki Valley had a significantly higher disappearance rate than other sites (Fig. 3.3). Lastly, disappearance rates were highest at Kahanahaiki Valley in all three treatments, and lowest at Palikea Trail (Fig. 3.3).

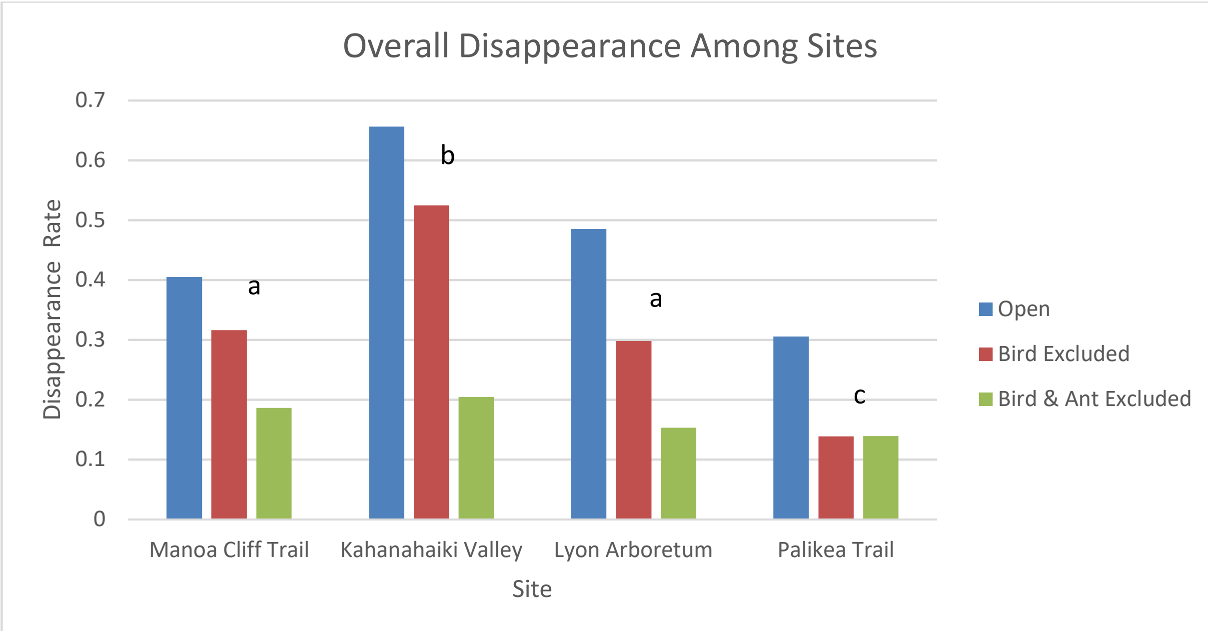

Figure 3.3. Disappearance rates among sites when all instars are pooled together. Identical letters indicate no significant difference between sites (Manoa Cliff and Lyon) based on binomial logistic regression. Different letters indicate significant differences after Bonferroni adjustment for multiple pairwise comparisons (p<0.008).

Kahanahaiki Valley

The effect of YCA at Kahanahaiki Valley can be seen in Fig. 3.4. For the treatments allowing ant entry, disappearance was significantly higher (<0.005) in the area containing YCA than in the area without the YCA. Furthermore, in the area containing YCA, there was no significant difference between the "open" and "bird excluded" treatment, but there was a highly significant (<0.005) difference between these two treatments and the "bird and ant" excluded treatment. Surprisingly, there was a slight significant difference ($p<0.05$) between the "bird and ant excluded" treatment of the two areas. Furthermore, in the area where the YCA is absent, there was a slight significant difference ($p<0.05$) between all three treatments.

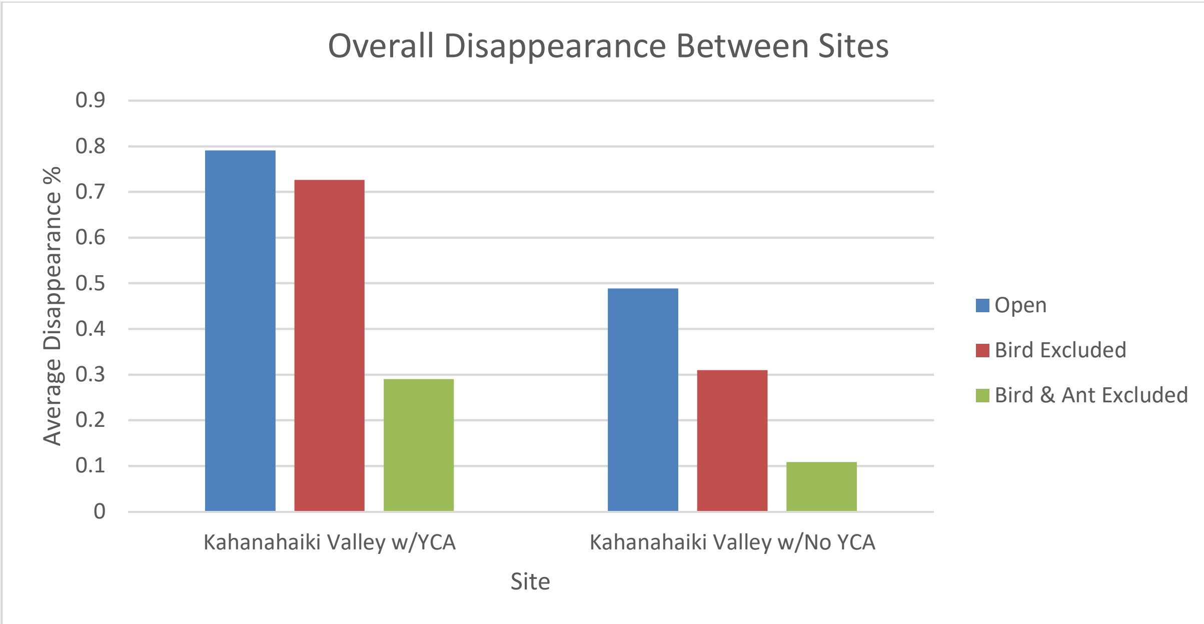

Figure 3.4. Average disappearance rates at Kahanahaiki of each treatment when all instars are pooled, between the two areas in Kahanahaiki Valley.

Risk-values (r_n)

Bird and Ant Effect

Risk-values provided an alternative way to explore the important threats faced by caterpillars and eggs throughout their life cycle. When looking at $A_{cumulative}$, Palikea Trail essentially had zero ant effect (Fig. 3.5). One site where the highly invasive YCA was present, Lyon Arboretum, showed the highest $A_{cumulative}$ of 0.25 (Fig. 3.5). Surprisingly, although the area in Kahanahaiki Valley containing YCA showed high disappearance in the treatments allowing ant entry (“open” and “bird excluded”; Fig. 3.4), the ant effect was relatively low, perhaps due to high disappearance of fifth instar caterpillars in the “bird and ant excluded” treatment (0.01; Fig. 3.5). This may be in part an artifact of low sample size in the “bird and ant excluded” treatment. Some sites where highly invasive ants were not detected, Manoa Cliff Trail and one area in Kahanahaiki Valley, still had a high $A_{cumulative}$ of 0.08 and 0.21, respectively, suggesting that crawling arthropod predators other than ants (e.g. spiders), may be important there (Fig. 3.5). On one occasion, a native *Mecaphesa* spider (Thomisidae) was observed feeding on a third instar caterpillar at Manoa Cliff Trail (Fig. 3.6). Although Palikea Trail had the lowest $A_{cumulative}$, it had the highest $B_{cumulative}$ of 0.10. It is possible that because ants were scarce at Palikea Trail, this allowed birds more time to search and predate the larvae. Conversely, it is possible that the areas containing YCA (Lyon Arboretum and Kahanahaiki Valley), had a low $B_{cumulative}$ because rapid ant predation masked the impact of birds. During one deployment, a third instar caterpillar was observed being attacked and carried away by a YCA within 15 minutes of being deployed (Fig. 3.7).

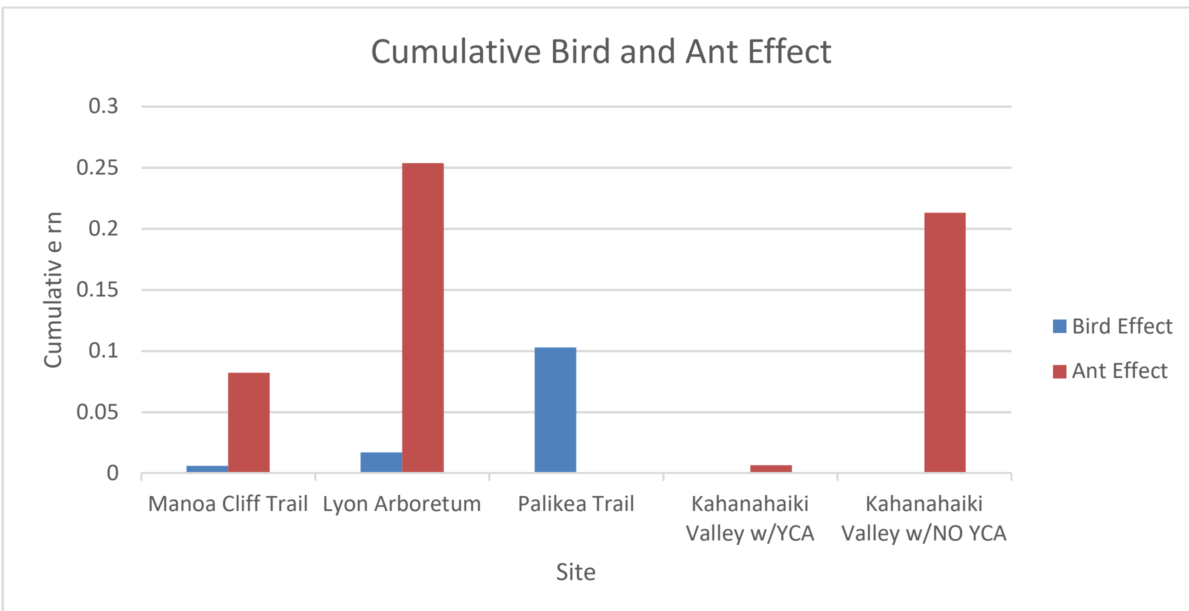

Figure 3.5. Cumulative r_n bird and ant effect of each site. Palikea Trail had the lowest ant effect and Lyon Arboretum had the highest ant effect.

Artificial Cohorts

Survival of artificial cohorts of 200 caterpillars was simulated using r_n values to visualize estimated mortality over the course of the life cycle if protected from birds and/or ants at each site. A cohort size of 200 was chosen because female Kamehameha butterflies generally lay between 100 and 300 eggs over their life spans in the laboratory (W. Haines unpublished data).

Kahanahaiki Valley was separated into two sites to compare the effect of YCA. When larvae are not protected, all sites show minimal survival by the fourth and fifth instars (Figs. 3.8 - 3.12). In the area of Kahanahaiki Valley with YCA, survival of unprotected larvae drops drastically by the second instar (Fig. 3.9). When larvae are protected from birds, predicted survival rates increase, but the majority of sites show minimal survival (fewer than 4 caterpillars) by the 5th instar with the exception of Palikea Trail (21 caterpillars). In the area of Kahanahaiki Valley invaded by YCA, the survival rate by the second instar when protected from birds more than doubles that of the survival rate when no protection is implemented, however by the third instar it drops to less than a single caterpillar (Fig. 3.9). Protection from both birds and ants showed the highest simulated survival rates. With protection from birds and ants, Lyon Arboretum had the highest simulated survival rate (54 caterpillars, Fig. 3.11), followed by the area in Kahanahaiki Valley without YCA (43 caterpillars, Fig. 3.10), Manoa Cliff Trail (18 caterpillars, Fig. 3.8), Palikea Trail (17 caterpillars, Fig. 3.12), and the area in Kahanahaiki Valley containing YCA (1 caterpillar, Fig. 3.9). When protection from birds and ants is simulated at Palikea Trail, where ants were not detected (Fig. 3.12), the overall survival rate by the fifth instar is similar to that when protected just from birds. This suggests that birds may be more important than crawling predators there. Likewise, at Lyon Arboretum (Fig. 3.11) and Kahanahaiki Valley without YCA (Fig. 3.10), the survival rates were similar with and without protection from birds, but when protected from birds and ants, survival increases drastically. This suggests that ants may be the important mortality factor at those sites.

Figure 3.6. *Mecaphesa sp*., native crab spider at Manoa Cliff feeding on a deployed third instar caterpillar.

Figure 3.7. *Anoplolepis gracilipes*, Yellow crazy ant at Kahanahaiki Valley feeding on a third instar caterpillar within 15 minutes of deployment.

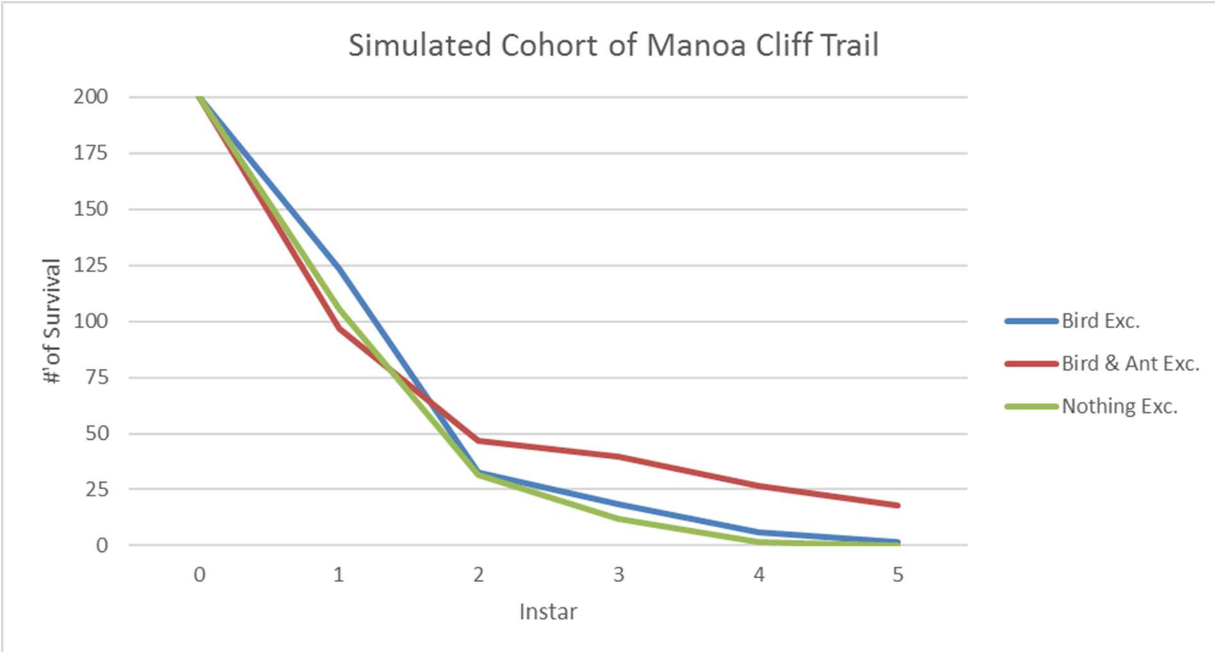

Figure 3.8. Simulated survival of an artificial cohort of 200 caterpillars at Manoa Cliff Trail when with and without protection from predators.

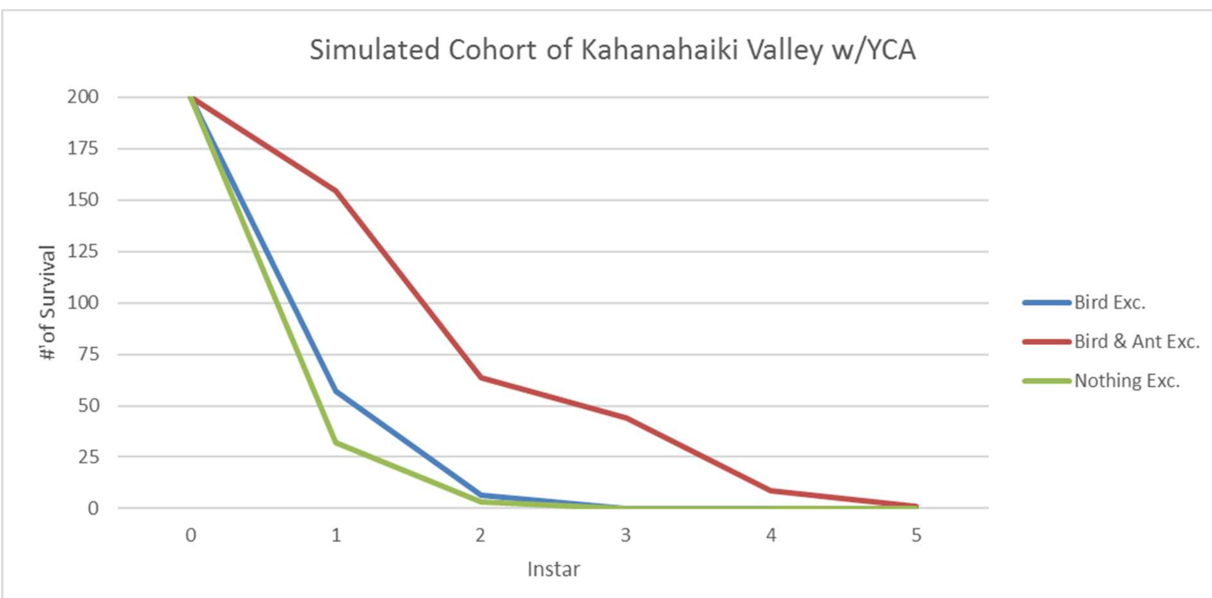

Figure 3.9. Simulated survival of an artificial cohort of 200 caterpillars at the Kahanahaiki Valley area invaded by YCA with and without protection from predators.

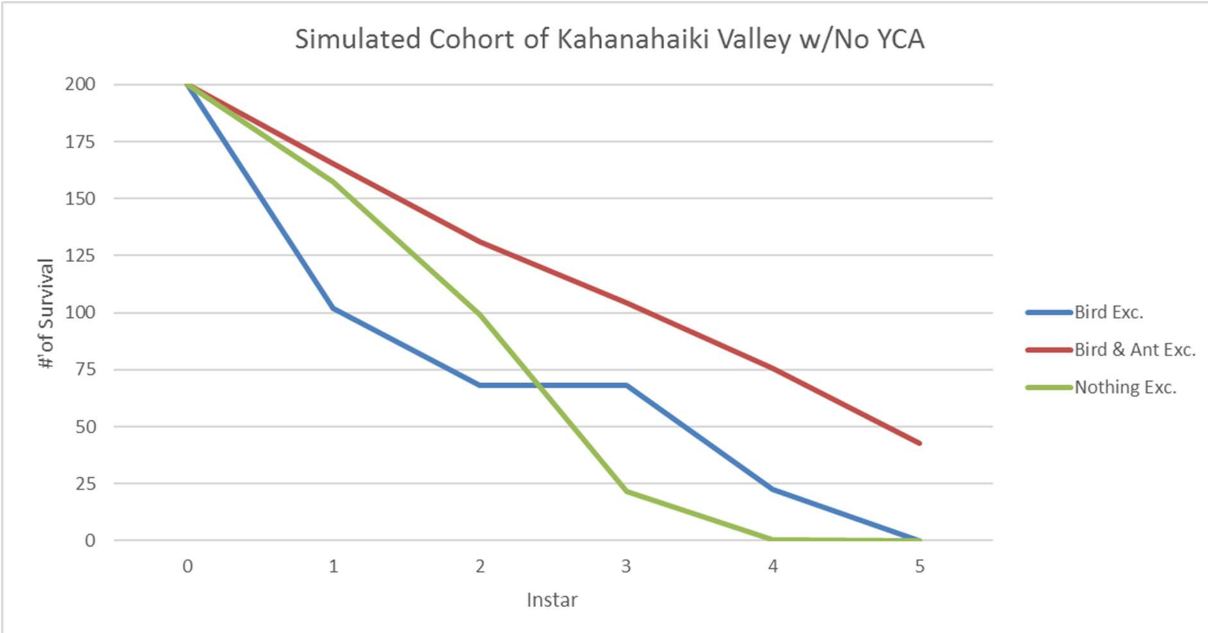

Figure 3.10. Simulated survival of an artificial cohort of 200 caterpillars at the Kahanahaiki Valley area uninvaded by YCA with and without protection from predators.

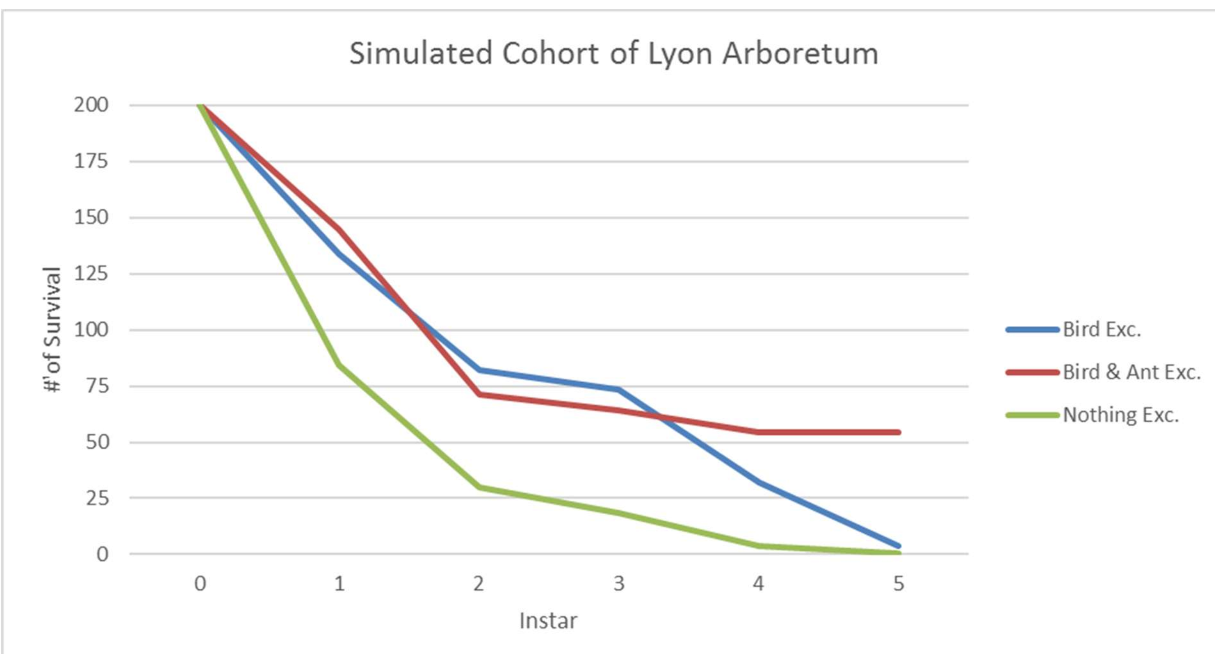

Figure 3.11. Simulated survival of an artificial cohort of 200 caterpillars at Lyon Arboretum with and without protection from predators.

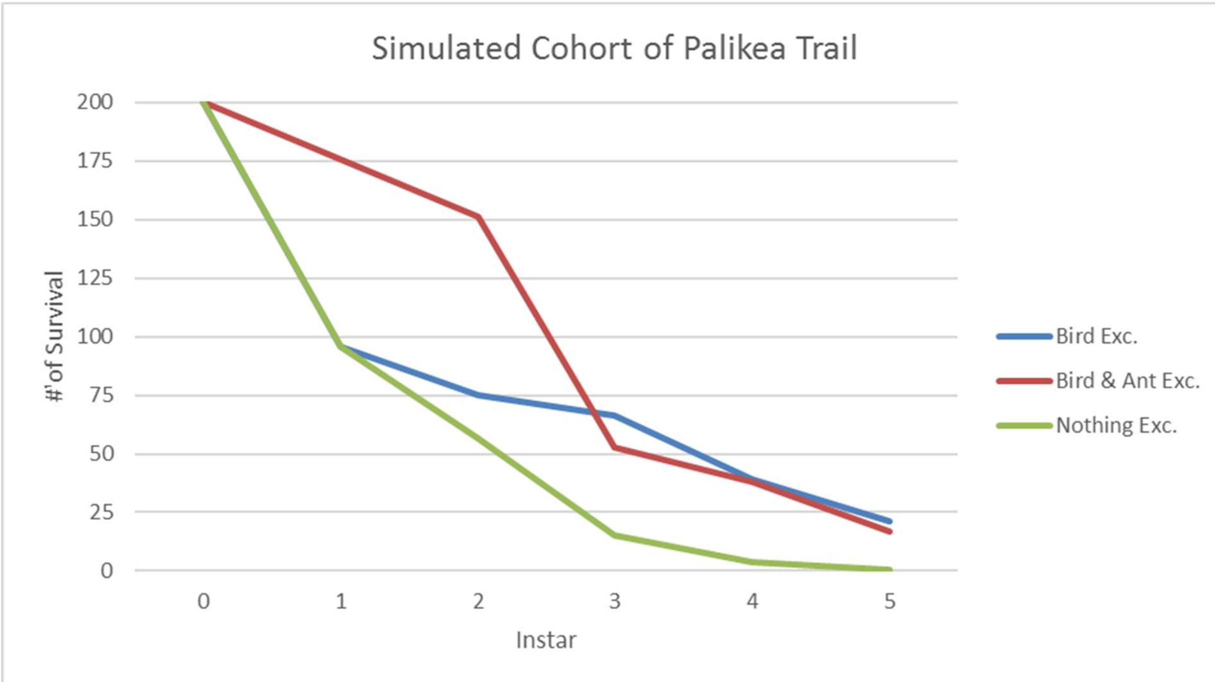

Figure 3.12. Simulated survival of an artificial cohort of 200 caterpillars at Palikea Trail with and without protection from predators.

Discussion

Egg and larval parasitism was only found at Palikea Trail, the highest and arguably least disturbed site. The absence of parasitism at Manoa Cliff Trail and Lyon Arboretum was somewhat expected, since the butterfly is no longer present there and parasitoid species of semi-concealed caterpillars were found to be more host specific than exposed caterpillars in one study in Papua New Guinea (Hrcek et al., 2013). However, recent studies have found parasitoids, including those introduced for biocontrol purposes, to infect other native Hawaiian Lepidoptera (Kaufman & Wright, 2009, 2010, 2011; King et al., 2010). The reason no generalist parasitoids were found to infect the caterpillars may be partially due to their leaf-folding behavior. The characteristic leaf-folding behavior of the Kamehameha butterfly larvae might offer protection against generalist parasitoids that are not adapted to searching for leaf-folds. Surprisingly, I did not find egg or larval parasitism at Kahanahaiki Valley, despite the presence of an extant natural butterfly population. The reason for this is unclear, but it may be due to elevation, and other ecological factors such as the presence of YCA and other ant species, which have been known to alter ecosystems (Krushelnycky et al. 2005; Krushelnycky 2015). Even at Palikea Trail, larval parasitism was negligible (0.72% [2/277] of caterpillars retrieved). The small number of larval parasitoids found coincides with observations of Williams (1928), and the absence of larval parasitoids in the records compiled by Leeper (2014) (Table 3.1). On the other hand, egg parasitism rates at Palikea varied, at times exceeding 80% (March 2016 deployment) and at other times being completely absent. It is possible that deployments were coincidentally timed with natural fluctuations in the parasitoid population, which may in turn be correlated with population fluctuations of the Kamehameha butterfly or other host species, as well as weather patterns. Studies conducted on other Lepidoptera species have found host population density to be an important factor correlated with parasitism by *Trichogramma* species (Reznik & Umarova, 1991). Despite the high egg parasitism rates detected during two deployments, the natural butterfly population continues to exist. Egg parasitism at Palikea Trail may represent a natural interaction and may not pose a significant threat to the population. DNA sequence data and morphological traits are consistent with the hypothesis that the egg parasitoid is a native *Trichogramma*, and if this is the case, the two species have likely evolved together. However, this host-parasitoid system warrants further investigation. Egg and larval parasitism does not seem to be a major limiting factor of the butterfly at most sites on Oahu. This is fortunate, since parasitoids would be difficult or impossible to manage once established in the wild.

It was suspected that generalized predators, including birds and ants, would cause higher impacts to deployed eggs and caterpillars at sites where butterflies have been extirpated. However, this was not necessarily the case. Despite hosting the butterfly, Kahanahaiki Valley had the highest larval disappearance in all three treatments (Fig. 3.3). It is unclear why this occurred, but it may be due in part to one area being infested with YCA, or low sample size and high migration, or simply because of a high abundance of predators there. Although Palikea Trail had the lowest “ant effect”, it had the highest “bird effect” (Fig. 3.5). This may be due to the lack of ants, permitting more time for the birds to find the larvae. It could also be because the butterfly still occurs at this site, along with other native larval species (e.g. Crambidae: *Udea stellata*) that feed on māmaki, inclining the birds to search for larvae on māmaki. Birds have been shown to alter their foraging behavior and search images based on the availability of prey items (Heinrich & Collins, 1983; Tvardikova & Novotny, 2012), so at sites where caterpillars are naturally present, birds may be accustomed to searching for Kamehameha butterfly caterpillars and shelters. Heinrich & Collins (1983) found that some birds keyed in on leaf morphology (leaf rolls and discoloration) to search for larvae, and others searched for caterpillars directly. The "bird effect" was lower than expected for the two sites lacking the butterfly, but birds may not be adapted to searching for caterpillars on māmaki trees there since the butterfly is no longer present there. If reintroductions are conducted at Lyon Arboretum and Manoa Cliff Trail, it is possible that the birds there may learn to recognize the leaf folding behavior and leaf damage, and key in on the larvae.

Palikea Trail was the only site that had a slightly higher disappearance rate in the "bird and ant excluded" treatment than the "bird excluded" treatment (Fig. 3.3). The difference in disappearance rates between the two treatments was negligible (0.1%). Because ants are not present at Palikea Trail, it is not surprising that once birds were excluded, adding protection from ants did not increase caterpillar survival (Fig. 3.12). Interestingly, Manoa Cliff Trail showed an "ant effect" even though no ants were detected there (Fig. 3.5), and caterpillars seemed to benefit from the ant-exclusion treatment (Fig. 3.8), suggesting that the ant barrier may have lowered disappearance rates by excluding other predators or by inhibiting migration. Other crawling predators, such as spiders, were observed (Fig. 3.6). Lyon Arboretum, as expected, showed a high ant impact (Fig. 3.5), most likely due to the presence of both YCA and the white-footed ant. In addition, Lyon Arboretum was the only site that showed high egg predation (Table 3.4). Being the lowest site and having high densities of orthopteran egg predators, this was not surprising.

To look at the impacts caused by YCA, Kahanahaiki Valley was separated into two sites for three analyses: overall disappearance (Fig. 3.4), artificial cohorts (Fig. 3.9, 3.10), and "bird and ant effect" (Fig. 3.5). In these comparisons, the area with YCA showed significantly higher disappearance in all three treatments (Fig. 3.4), and both areas had the lowest simulated survival to pupation of all of the sites (Table 3.6). Interestingly, there was a significant difference in disappearance rates in the "bird and ant excluded" treatments between the two areas (Fig. 3.4). This may be because the area containing YCA is more open to the weather elements or there may be more flying insect predators that can bypass the exclosure. Both the "bird effect" and “ant effect” was minimal in the area containing YCA, which was surprising since the treatments allowing ant entry (“open”, “bird excluded”) had significantly higher disappearance rates than that of the “bird and ant excluded” treatment in that area (Fig. 3.4). Likewise, although disappearance rates were significantly lower in the area without YCA, the “ant effect” was much higher than that of the area containing YCA. It is unclear why the “ant effect” was so high, but it could have been caused by the presence of other ant species or crawling predators (e.g. spiders, predaceous nabid bugs) in that area. Low sample size of each instar may be to blame for the unforeseen “bird and ant effect” in the two areas.

Table 3.6. Risk-values (r_n values) of each instar at each site, and simulated survival of artificial cohorts of 200 caterpillars with and without protection from specific predators.

Site	Butterfly	Instar	Bird & Ant Exc. r_n	Bird Exc. r_n	Open r_n	Effect of Birds r_n	Effect of Ants r_n	Cohort 200 Bird & Ant Exc.	Cohort 200 Bird Exc.	Cohort 200 Nothing Exc.
Manoa Cliff Trail	**No**	1	0.516	0.383	0.471	0.088	0.000	96.9	123.4	105.7
		2	0.516	0.737	0.701	0.000	0.221	46.8	32.4	31.6
		3	0.159	0.433	0.619	0.186	0.274	39.4	18.4	12.0
		4	0.323	0.673	0.857	0.183	0.350	26.7	6.0	1.7
		5	0.333	0.773	0.886	0.113	0.439	17.8	1.4	0.2
		Cumulative	0.911	0.993	0.999	0.006	0.082			
Lyon Arboretum	**No**	1	0.277	0.330	0.578	0.248	0.053	144.6	134.0	84.5
		2	0.508	0.388	0.649	0.261	0.000	71.2	82.0	29.6
		3	0.099	0.103	0.380	0.277	0.003	64.1	73.6	18.4
		4	0.152	0.567	0.794	0.227	0.416	54.4	31.8	3.8
		5	0.000	0.885	0.930	0.045	0.885	54.4	3.7	0.3
		Cumulative	0.728	0.982	0.999	0.017	0.254			
Palikea Trail	**Yes**	1	0.121	0.522	0.522	0.000	0.401	175.9	95.6	95.6
		2	0.141	0.216	0.412	0.195	0.076	151.2	74.9	56.3
		3	0.651	0.114	0.735	0.621	0.000	52.7	66.4	14.9
		4	0.281	0.408	0.745	0.337	0.126	37.9	39.3	3.8
		5	0.561	0.462	0.844	0.382	0.000	16.6	21.1	0.6
		Cumulative	0.917	0.894	0.997	0.103	0.000			
Kahanahaiki Valley w/YCA	**Yes**	1	0.228	0.714	0.839	0.125	0.486	154.5	57.3	32.2
		2	0.588	0.883	0.901	0.018	0.295	63.6	6.7	3.2
		3	0.306	0.974	0.941	0.000	0.668	44.2	0.2	0.2
		4	0.804	0.975	0.999	0.024	0.172	8.7	0.0	0.0
		5	0.850	1.000	1.000	0.000	0.150	1.3	0.0	0.0
		Cumulative	0.993	1.000	1.000	0.000	0.007			
Kahanahaiki Valley w/NO YCA	**Yes**	1	0.173	0.491	0.214	0.000	0.318	165.4	101.7	157.2
		2	0.206	0.329	0.370	0.041	0.123	131.3	68.3	99.0
		3	0.206	0.000	0.780	0.780	0.000	104.3	68.3	21.8
		4	0.274	0.672	0.971	0.299	0.398	75.7	22.4	0.6
		5	0.436	0.995	1.000	0.005	0.559	42.7	0.1	0.0
		Cumulative	0.787	0.999	1.000	0.001	0.213			
Kahanahaiki Valley Combined	**Yes**	1	0.145	0.610	0.627	0.017	0.464	170.9	78.1	74.6
		2	0.452	0.666	0.710	0.044	0.214	93.8	26.1	21.7
		3	0.263	0.680	0.887	0.207	0.417	69.1	8.4	2.4
		4	0.590	0.877	0.991	0.114	0.287	28.3	1.0	0.0
		5	0.745	1.000	1.000	0.000	0.255	7.2	0.0	0.0
		Cumulative	0.964	1.000	1.000	0.000	0.036			

An issue encountered during the analyses process was low sample size when data was broken down to finer comparisons. For example, when looking at the area of Kahanahaiki Valley without YCA, 206 total larvae were deployed (77 “open” treatment, 55 “bird and ant excluded” treatment, and 74 “bird excluded” treatment), and of those, only 23 were fifth instars. Eight (8) fifth instars were deployed in the “open” and “bird and ant excluded” treatment, and seven (7) in the “bird excluded” treatment. When all instars were pooled and treatments were compared (Fig. 3.4), clear broad trends can be seen, and these generally match expectations. However, when the disappearance rates were broken down to look at each instar, in each treatment, at each site, like in the simulated cohort survival (Fig. 3.10), a high percentage of disappearance of one small subset of caterpillars can have a large effect on the resulting survivorship curve or cumulative r_n. For instance, a disappearance rate of 71% (5 of 7) for the fifth instar in the “bird excluded” treatment would have shown an extremely high “ant effect” (despite there being no YCA) since there was only a 13% (1 of 8) disappearance rate for the same instar in the “bird and ant” excluded treatment. A higher sample size should be conducted to further investigate rates of bird and ant predation on specific instars at each site.

Larval disappearance caused by predation was difficult to segregate from caterpillar migration. The ant-exclusion treatment likely limited caterpillar migration, although it did not prevent caterpillars from dispersing by dropping off the foliage, which was often seen in wild and captive larvae. However, as mentioned in Chapter 2, instars one through four exhibit leaf-folding behavior, and migration appeared to be typically low in these instars. In the fifth instar, caterpillars cease constructing distinct shelters and tend to migrate more often to other branches to satisfy their large appetite. At all sites, migration (and the prevention of it) is likely to have had a much larger confounding effect on disappearance rates of the fifth instar than on earlier instars. 268 total fifth instar caterpillars were deployed across the four sites, and only 59% were retrieved. This was the lowest retrieval rate for all instars, except the fourth, which also had a retrieval rate of 59%. It is possible that fourth instars, especially as they matured, migrated more frequently than earlier instars. The high disappearance in the fourth and fifth instars could have been due to predation, but this trend was not expected to be seen in the “bird excluded” treatment, since bird predation (at sites with no ants detected) was expected to be the main cause of disappearance for the older larvae. At Manoa Cliff Trail, for instance, although no ants were detected, the “bird excluded” treatment followed the same trend as the “open” treatment. Likewise, the area of Kahanahaiki Valley without YCA, had a low survival in both the “open” and “bird excluded” treatment (Table 3.6). Sites containing YCA, could have experienced predation in the later instars, since YCA is known to kill organisms much larger than themselves (Abbott, 2006). Migration could have also affected the disappearance seen in the “bird and ant” excluded treatment. Even though the larvae were protected from all predators, there was still low recovery at all of the sites. Two explanations for this, other than migration by dropping off vegetation, is if the caterpillar crawled on or outside the bird-netting, negating its protection, or if flying and jumping insects got into the treatment. Both of these potential causes of disappearance, in combination with migration, could have resulted in the high disappearance seen in the “bird and ant excluded” treatment.

Segregating egg disappearances due to predation from those due to weather was difficult. Eggs were only considered predated when there were obvious chewing marks, and when no hatchlings or feeding damage were found on the same leaf. Eggs and wax paper that were completely missing were generally presumed to be results of wet weather or human error (e.g. insufficient glue). During deployments, eggs were difficult to glue to moist leaves. A dry cloth was used to attempt to dry leaves before gluing, but glue likely took much longer to dry under wet and humid conditions. It is also possible that even dry glue could have separated from the leaves if subjected to heavy rain and wind, which often occurred at the study sites.

After analyzing the data, it is apparent that predators like birds and ants are important threats to the Kamehameha butterfly on Oahu. This is especially apparent when comparing overall disappearance rates among treatments at each site (Fig. 3.3). Surprisingly, when artificial cohorts of unprotected larvae were simulated, all sites, including those that currently support natural populations of the butterfly, showed minimal survival to pupation. However, it is important to note that these are only estimates and are mainly meant to compare relative survival between treatments at each site. It is difficult to translate these numbers to absolute numbers of survivors, but it gives an idea of what actions might be taken to protect caterpillars, particularly if reintroductions are attempted. It is also important to note that females in the wild are subjected to predation, and the mean number of eggs laid by wild females is unknown, though females in captivity have been found to lay up to 300 eggs over the course of their approximately 30 day lifespan (W. Haines unpublished data). Simulated cohorts with larvae protected from birds alone and from both birds and ants showed higher survival rates at all sites (Figs. 3.8 – 3.12), suggesting that all sites could benefit from protection of immature stages.

Although only a few top-down limiting factors were studied, data suggests that larval predation by birds and ants are more important threats than larval parasitism on Oahu, and sites that currently do not support the butterfly (Manoa Cliff Trail and Lyon Arboretum), might establish a population if certain controls are implemented in combination with reintroductions. Two ways to control birds could include physically removing them or placing bird netting over entire māmaki trees or branches. However, one issue with bird netting is the likelihood of deterring oviposition by adults. Ants should also be controlled, by either applying physical ant barriers at the base of māmaki trees or using chemical treatments such as formicidal baits. These protective measures can be adapted by homeowners, community gardens, and other managed areas. Managers of natural areas considering ant and bird management, along with the reintroduction of Kamehameha butterflies, might explore the feasibility of physically controlling birds and chemically controlling ants, as these may provide long-term effects. However, non-target effects of control methods on native invertebrates or other animals must be considered, especially when applying pesticides in natural areas.

In addition to protecting eggs and caterpillars from predators, it may be beneficial to encourage planting of butterfly host plants in appropriate areas to help the butterfly expand its range. Māmaki has been documented to inhabit mesic to wet forests ranging in elevation from 200-6000 feet on all Hawaiian Islands except Niihau and Kahoolawe (Pratt, 2009; Little Jr. & Skolmen, 1989). Currently, it is being propagated by various farms around Hawaii for herbal tea preparations (Kartika et al., 2011). The plant is easy to grow as long as it receives adequate moisture, and it can be propagated by seed and cuttings (Bornhorst, 2005). However, great consideration should be taken when planting larval host plants, as it is possible that outplantings can have a negative effect if they create low-quality habitat. For example, one study done on the pipevine swallowtail, *Battus philenor* L. (Papilionidae), found that some gardens in California containing the larva's host plant, *Aristolochia californica* Torr (Aristolochiaceae), acted as a population sink (Levy & Connor, 2004). Although the butterfly successfully oviposited on garden host plants, the density and survival rate of the eggs were much lower than that of eggs laid at natural sites. Similar conclusions regarding population sinks were reached after two butterfly reintroduction studies were conducted (van Langevelde & Wynhoff, 2009; Adamski & Witkowski, 2007). The same outcome could occur if māmaki are planted, but are not protected from predators, or if they are planted in areas that are unsuitable for other reasons. For example, although the butterfly is extant in Kahanahaiki Valley, the overall disappearance of the two areas combined (Fig. 3.3) suggests that the butterfly should not be able to survive there. Being that YCA is only present in part of the study site, this invaded area might be acting as a sink (Dias, 1996), and the butterfly population might not persist if the entire site were invaded by YCA. If the Kamehameha butterfly lays its eggs on unprotected plants in an area (like Kahanahaiki Valley with YCA) where egg and larval predators (e.g. birds, ants, katydids) are abundant, its offspring may not survive, having a net negative effect on the population as a whole. It is crucial that habitat quality, including protections from predators, is carefully considered when planting the host plants of the Kamehameha butterfly.

Protecting the immature stages of the Kamehameha butterfly from birds and ants should be among the highest priorities when trying to establish populations in new areas or when trying to expand a current population through active management. Other limiting factors such as different predator and parasitoid assemblages, temperature, elevation, weather, host plant abundance, and pathogens, may be important, so these should also be studied prior to or in conjunction with any reintroduction attempts. However, if nonnative birds and ants are indeed found to be the primary threats to the Kamehameha butterfly, the butterfly should benefit from efforts to control these predators and prevent introduction of additional nonnative species.

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